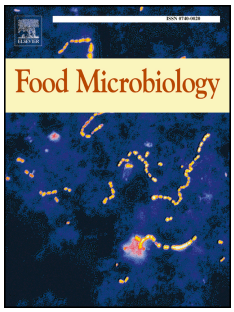

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Vincenzo Fallico, Mary Rea, Catherine Stanton, Niclas Ilestam, Julie McKinney

PII: S0740-0020(20)30090-3

DOI: https://doi.org/10.1016/j.fm.2020.103501

Reference: YFMIC 103501

To appear in: *Food Microbiology*

Received Date: 12 July 2019

Revised Date: 4 December 2019

Accepted Date: 1 April 2020

Please cite this article as: Fallico, V., Rea, M., Stanton, C., Ilestam, N., McKinney, J., Next-generation multiparameter flow cytometry assay improves the assessment of oxidative stress in probiotics, *Food Microbiology* (2020), doi: https://doi.org/10.1016/j.fm.2020.103501.

SHORT COMMUNICATION

Next-generation multiparameter flow cytometry assay improves the assessment of oxidative stress in probiotics

Vincenzo Fallico[1#], Mary Rea[1], Catherine Stanton[1], Niclas Ilestam[2*], Julie McKinney[3^]

[1]Teagasc Moorepark Food Research Centre, Fermoy, County Cork, Ireland

[#]current address: DuPont Nutrition & Biosciences, Madison, Wisconsin, USA

[2]Pfizer Consumer Healthcare, Søborg, Gribskov, Denmark

[*]current address: BioGaia AB, Stockholm, Sweden

[3]Pfizer Consumer Healthcare, Richmond, Virginia, USA

[^]current address: Reckitt Benckiser, Montvale, New Jersey, USA

ABSTRACT

Stability of probiotic products' potency throughout shelf life is essential to ensure systematic delivery of the dosages required to provide clinically-proven health benefits. Due to the oxygen sensitivity of gut-derived microorganisms, methods for the rapid and accurate monitoring of oxidative stress in probiotics are greatly needed as they can be instrumental to both bioprocess optimization and quality control. This study introduces a next-generation flow cytometry method multiplexing the CellROX® Green and Propidium Iodide probes for the simultaneous measurement of free total reactive oxygen species (ROS) and membrane integrity, respectively. The multiparameter method was compared to the single-parameter assays, measuring either ROS or membrane integrity, for the ability to evaluate the fitness of *Lactobacillus rhamnosus* GG (LGG) after freeze drying, spray drying and H_2O_2-mediated oxidative stress. Each stand-alone assay detected only three cell populations, showing either differential membrane integrity (Syto 24+/PI-, Syto 24+/PI+, Syto 24-/PI+) or ROS levels (ROS-, low-ROS, high-ROS), and no correlation could be drawn between these groups. Conversely, the multiparameter method detected up to five physiologically distinct cell populations and allowed the integrated assessment of their membrane integrity and oxidative stress. It also revealed a much larger fitness heterogeneity in LGG as each group of low-ROS and high-ROS cells was found to be formed by a healthier population with an intact membrane (L-ROS/PI-, H-ROS/PI-) and a population with damaged membrane (L-ROS/PI+, H-ROS/PI+). As the CRG probe only detects free unreacted ROS, these populations are suggested to reflect the dynamic lifecycle of ROS formation, accumulation and reactive depletion leading to oxidative damage of macromolecules and consequent cell death. With the stand-alone CRG assay being unable to detect ROS lifecycle, the multiparameter method

here presented delivers a superior profiling of the heterogeneity generated by oxidative stress in bacteria and enables a more correct interpretation of CRG fluorescence data. We provide recent examples from literature where the use of a single-parameter fluorescence approach may have led to misinterpret oxidative stress data and eventually draw erroneous conclusions.
Keywords: Probiotics, Oxidative stress, Membrane integrity, Flow cytometry

1. Introduction

Functional foods and supplements containing health-promoting probiotics are rapidly becoming one of the most popular categories of bioactive products in the wellness market, as consumers search for alternative, drug-free approaches to maintain or enhance personal well-being. Supported by clinical studies detailing their health benefits (O'Bryan et al., 2013; Hill et al., 2014), the global market of probiotics has exceeded USD 34 billion in 2015 and is expected to be worth USD 64 billion by 2023 (GMI, 2016). The accepted definition of probiotics is that of microorganisms able to provide health benefits to the host, beyond general nutrition, when ingested live and in adequate amounts (FAO/WHO, 2002). With growing demands to be met and diverse geographical markets to be reached, the main commercial challenge is to manufacture high-potency, storage-stable probiotic products ensuring, throughout their shelf-life, the systematic delivery of the health benefits outlined in the clinical studies to consumers worldwide.

Probiotics are commercially dried into highly concentrated powders as this convenient and inexpensive format facilitates both formulation into functional foods as well as storage, handling and distribution without the need for specialized refrigerated containers (Muller et al., 2009). However, several factors can affect probiotics' viability during manufacturing and shelf-life, with oxidative stress being a major cause of cell mortality due to the oxygen-sensitive nature of these gut-inhabiting microbes (Upadrasta et al., 2011). Healthy cells use a battery of enzymatic and non-enzymic detoxification systems to maintain intracellular homeostasis of highly reactive and unstable free radicals, termed reactive oxygen species (ROS), which are generated by the regular bacterial metabolism (e.g., enzymatic, redox and Fenton reactions). However, manufacturing and storage conditions can cause the intracellular ROS levels to exceed this scavenging capacity and result in oxidative damage of membrane lipids, proteins and nucleic acids (Zhao and Drlicab, 2014). The connection between oxidative reactions and bacterial instability upon drying and storage has been clearly demonstrated. Survival to spray drying was improved when either an antioxidant was included in the drying medium or nitrogen replaced air as the atomizing gas (Ghandi et al., 2012). During storage, dried bifidobacteria and lactobacilli survived significantly better in the presence of antioxidants or when the environmental oxygen was removed using either nitrogen or vacuum (Celik and O'Sullivan, 2013; Kurtmann et al., 2009; Hsiao et al., 2004). Consequently, methods for the rapid and accurate assessment of oxidative stress in probiotics are of great industrial importance as they can assist bioprocess groups in optimizing strategies

to minimize oxygen's toxicity and quality control teams in better profiling probiotics fitness in raw and finished products.

Used alone or in combination with fluorescent probes, flow cytometry (FC) is a powerful and rapid technique enabling single-cell analysis of a wide range of structural and functional cell properties. FC can provide a valuable support to bioprocess design and control through the in-depth characterization of the physiological heterogeneity existing within microbial populations (Diaz et al., 2010). In terms of oxidative stress analysis in bacteria, several fluorescent dyes have been used to detect, with ranging specificity, individual ROS such as H_2O_2, hydroxyl radicals or superoxide anions (Dwyer et al., 2014; Keren et al., 2013). Alternatively, total ROS levels can be measured using an unspecific probe such as the cell-permeable, non-fluorescent CellROX® Green (CRG) reagent, which exhibits strong green fluorescence upon oxidation from ROS. By detecting any ROS type only when in their free unreacted form, the CRG probe measures oxidative stress rather than oxidative damage (Choi et al., 2015). As oxidative stress can provoke cell death via damage of membrane lipids and proteins, the rapid analysis of membrane integrity is undoubtedly important in assessing probiotics fitness. In FC, this is classically achieved by combining two nucleic acids-intercalating dyes, such as the membrane-permeant Syto 9 and the membrane-impermeant counterstain Propidium Iodide (PI). Live cells with an intact membrane will only be stained by Syto 9, dead cells with a compromised membrane will only be stained by PI due to its higher affinity for nucleic acids than Syto 9, whereas injured cells will uptake and retain both dyes (Shapiro, 2015).

To the best of our knowledge, only fluorescent methods analysing either oxidative stress or membrane integrity in bacteria have been reported to date. In this study, we show the limitations of using a single-parameter approach to assess oxidative stress and introduce a next-generation multiparameter FC assay enabling the simultaneous detection of free total ROS via the CRG sensor with PI-mediated assessment of membrane permeability. Compared to the stand-alone ROS assay, the multiparameter method provides a better depiction of ROS life cycle by discriminating between the stages of ROS formation, accumulation and reactive depletion. This delivers a superior accuracy in profiling the oxidative stress heterogeneity in bacteria and greatly facilitates the interpretation of fluorescence-based data. We finally discuss how the inability of end-point single-parameter fluorescent analysis to fully document ROS life cycle could have ignited the recent controversy regarding ROS involvement in antibiotic lethality (Keren et al., 2013; Dwyer et al., 2014).

2. Materials and Methods

2.1 Bacterial strains and media

Frozen pellets of the probiotic bacterium *Lactobacillus rhamnosus* GG (LGG) were obtained from Chr. Hansen (Hoersholm, Denmark). LGG was propagated in MRS medium (Difco, UK) at 37ºC under microaerophilic conditions created using Anaerocult C bags (Merck Millipore, Ireland). Bacterial stock cultures were prepared in 15% glycerol and stored at -80ºC.

2.2 Preparation, drying and storage of probiotic powders

For spray-drying experiments, frozen pellets of LGG were re-suspended at 2% (w/v) into sterile 20% (w/v) reconstituted skim milk (RSM) (Kerry Ingredients, Ireland), supplemented or not with 0.05% cysteine. The probiotic suspensions were tempered at 37ºC for 30 min using a water bath before being spray-dried at an inlet temperature of either 140ºC or 170ºC and an outlet temperature of 85ºC using the Buchi Mini Spray Dryer B-191 (Buchi Labortechnik, Switzerland). Five-grams portions of each batch (140ºC or 170ºC) of spray dried powders were either vacuum-packed (VP) or non-vacuum packed (NVP) into TFL1 multilayer (polyester/aluminium/polyethylene) moisture barrier foil pouches (Faulkner Export Packaging, Ireland). Spray dried powders were stored at 37ºC with no control of relative humidity and analysed after 8 months of storage by using the multiparameter FC assay and plate counts.

For freeze-drying experiments, frozen pellets of LGG were re-suspended at 2% (w/v) into 7 different sterile formulations (FD1-FD7) containing various combinations and amounts of RSM, MPC80 (Glanbia Nutritionals, Ireland), trehalose (Cargill, US), Orafti P95 (BENEO Orafti, Belgium), HyPea (Kerry Biosciences, US), lactose and glutathione (Sigma, Ireland). The probiotic suspensions were transferred to stainless steel trays and freeze-dried using the VirTis® AdVantage™ benchtop freeze dryer (SP Industries, USA). Freezing was performed by setting the shelf temperature at -40ºC. Primary drying was obtained at a shelf temperature of -25ºC followed by stepwise increases to -10ºC, 0ºC and +4ºC. Secondary drying was achieved at a shelf temperature of +10ºC. Five-grams portions of each FD1-FD7 formulation were VP into TFL1 foil pouches and stored at 37ºC with no control of relative humidity, except for FD3 that was also stored at 4ºC with no control of relative humidity. All freeze-dried samples were analysed after 5 months of storage by using the multiparameter FC assay and plate counts.

Spray-drying, freeze-drying and storage stability experiments were performed in duplicate.

166

167 **2.3 Freeze-Thaw-Desiccation challenge**

168 LGG from a 15% (w/v) glycerol stock was propagated at 37ºC for 18 h in MRS broth
169 supplemented with 0.05% cysteine. Cells were recovered by centrifugation (2,790 g for 5
170 min), washed using sterile Maximum Recovery Diluent (MRD) (Oxoid, UK), re-suspended to
171 1/100 of the original volume in sterile formulations of either 3% or 4% inosine supplemented
172 or not with 0.05% cysteine, and left to equilibrate at room temperature for 30 min. Samples
173 were frozen at -20°C for 18 h, thawed at 37°C for 30 min using a water bath, and desiccated
174 as follows. 0.2 ml of cells were mixed with 0.2 ml of 90% (w/w) glycerol and incubated at
175 room temperature for 15 min. After this, the cell suspension was subjected to four more
176 rounds of addition of 0.2 ml of 90% (w/w) glycerol, brief mixing by vortex and incubation at
177 room temperature for 15 min. This protocol resulted in exposure of cells to incremental
178 concentrations (45% to 90%) of glycerol and aimed to generate a rapid proxy of the
179 progressive dehydration occurring in cells during freeze-drying (Mille et al., 2004). Frozen-
180 thawed-desiccated (FTD) cells were recovered by centrifugation (20,000 g for 2 min),
181 washed using MRD, re-suspended in the corresponding formulation and stored at 4°C before
182 being used for growth curves and FC analyses. FTD trials were performed in duplicate.

183

184 **2.4 Viability analysis of LGG powders**

185 Two biological replicates were performed for each sample as follows. 1 gr of LGG powder
186 was aseptically resuspended in 9 ml of sterile MRD by vortex mixing for 1 min, followed by
187 30 min rehydration at room temperature and vortex mixing for 1 min. Ten-fold serial
188 dilutions of powder suspensions were prepared in MRD, pour plated using MRS agar and
189 incubated at 37°C for 2 days under microaerophilic conditions created using Anaerocult C
190 bags (Merck Millipore, Ireland). Plates containing 30-300 colonies were used to calculate cell
191 viability, which was expressed as colony forming units (CFU) per gr of sample.

192

193 **2.5 Growth curves analysis of FTD LGG**

194 Two biological replicates were performed for each sample as follows. FTD LGG cells
195 were aseptically inoculated at 2% (v/v) into 200 µl of MRS containing 0.05% cysteine
196 (MRSC) and of MRSC supplemented with 8% (w/v) NaCl to determine the impact of FTD
197 challenge on cell viability and membrane integrity, respectively. The kinetics of cellular

growth at 37°C were calculated by measuring samples absorbance at 590 nm at intervals of 1 h over 22 h using the microplate reader GENios plus (Tecan Trading, Switzerland).

2.6 Multiparameter FC analysis of LGG powders and FTD cultures

LGG powders (1 gr) were aseptically rehydrated in 9 ml of 0.2 μm-filtered MRD at room temperature for 30 min. One ml of each powder suspension or FTD culture was ten-fold diluted in 0.2 μm-filtered MRD containing 0.02% (v/v) Tween 20 (Invitrogen, Ireland) and 1 mM EDTA (Sigma, Ireland) to an appropriate cell concentration allowing for the analysis of ca. 1,000 events per sec and avoidance of cells coincidence at the flow cytometer.

Uniparameter analysis of oxidative stress was carried out by staining bacterial samples with 0.5 μM CRG (Molecular Probes, USA) for 30 min at 37°C, using a heating block with lid and a desiccator bag to minimize sample exposure to light and oxygen. Single-parameter analysis of membrane integrity was carried out by staining bacterial samples with 6.68 μM Syto 9 and 40 μM PI from the Live/Dead® BacLight™ viability kit (Molecular Probes, USA) for 15 min at 37°C, under agitation (400 rpm) in the dark.

Multi-parameter analysis of oxidative stress and membrane integrity was carried out by incubating bacterial samples first with 0.5 μM CRG for 15 min at 37°C, using a heating block with lid and a desiccator bags to minimize sample exposure to light and oxygen. Upon completion, bacterial samples were added of 8 μM PI and incubated for further 15 min under the same conditions as per CRG staining.

Stained samples were acquired for 1 min at medium flow rate (35 μl/sec) using a BD Accuri C6 flow cytometer (BD, Belgium) and a threshold set on forward scatter (6,000 for powders; 16,000 for cultures) to minimize background. Data acquisition and analysis were carried out using the BD Accuri C6 software v. 1.0.2 (BD, Belgium). The bacterial population was identified and gated on forward scatter (FSC) versus side scatter (SSC) biplots to filter background particles out from fluorescence analysis. The green fluorescence of Syto 9^+ or CRG^+ cells was monitored on the FL1 channel (530/30 nm) whereas red-fluorescent PI^+ cells were detected on the FL3 channel (> 670 nm long pass). Results for each gated subpopulation were expressed as percent cell events compared to the events of the total cell population.

3. Results and Discussion

3.1 A novel multiparameter FC assay improves oxidative stress analysis in probiotics

In this study, we set forth to leverage FC for the rapid, single-cell assessment of physiological parameters relevant to probiotics health such as oxidative stress and membrane integrity. While multicolour FC is an established routine in clinical research, most of the FC assays developed for microbial cells tend to measure one parameter at a time (Diaz et al., 2010). Figure 1 summarizes the analytical outcomes of using single-parameter FC formats measuring either oxidative stress or membrane integrity to evaluate the fitness of the probiotic strain model LGG after freeze drying. Using the oxidative stress sensor CRG, three populations containing varying levels of total ROS were detected in dried LGG (Figure 1A). The population emanating CRG fluorescence below $7x10^2$ arbitrary units (AU) was deemed ROS^- as unstained cells showed similar levels of background fluorescence (results not shown). ROS^+ cells exhibited CRG fluorescence above this threshold and formed two populations characterized by either low or high levels of free ROS. When the classical dual staining measuring membrane integrity as viability biomarker was used, three physiologically distinct populations, corresponding to live (Syto $9^+/PI^-$), injured (Syto $9^+/PI^+$) and dead (Syto $9^-/PI^+$) cells, were again detected in dried LGG (Figure 1B). The above results suggest that no more than three stages of cellular fitness can be discriminated using the single-parameter FC approach. Another major drawback is also the inability to clearly correlate the populations mirroring the integrity status of the cellular membrane to those containing varying loads of ROS.

To overcome these limitations, we multiplexed the total ROS sensor CRG with the membrane permeability probe PI to develop a next-generation multiparameter FC method that proved superior to each stand-alone assay on several aspects. First, a much larger physiological heterogeneity was revealed to exist in freeze dried LGG as five cell populations could now be clearly discriminated (Figure 1C). Most importantly, multiplexed staining allowed for the combined and unambiguous assessment of the membrane integrity and oxidative stress status of each cell population. It was now revealed that each group of low-ROS (L-ROS) and high-ROS (H-ROS) cells can be formed by two populations with very distinct health status possessing either an intact (L-ROS/PI^-, H-ROS/PI^-) or a damaged (L-ROS/PI^+, H-ROS/PI^+) membrane. Similarly, both dead cells (N-ROS/PI^+) and cellular debris (N-ROS/PI^-) can potentially contribute to the ROS^- population detected by the stand-alone ROS assay (Figure 1A).

To confirm these initial results and the physiological status of these newly identified populations, commercial frozen pellets of LGG were exposed stepwise to processes triggering oxidative stress and changes in cell fitness were analysed using the stand-alone

ROS assay and the multiparameter method. Pre-stress LGG pellets mainly contained L-ROS cells (Figure 2-A1), which were mostly healthy with an intact membrane (L-ROS/PI^-) whereas only a small fraction showed membrane damage (L-ROS/PI^+) (Figure 2-B1,C1). Low amounts of H-ROS cells were also present and formed by two equal populations with either an intact (H-ROS/PI^-) or a damaged (H-ROS/PI^+) membrane. Finally, dead cells (N-ROS/PI^+) and debris (N-ROS/PI^-) contributed equally to the ROS^- population (Figure 2-B1, C1). LGG frozen pellets were re-suspended into RSM supplemented with cysteine and stressed by spray drying, a process where bacterial death is mostly due to oxidative stress during the atomization stage rather than to desiccation (Ghandi et al., 2012). As expected, spray drying generated extensive oxidative stress and membrane damage in the initially healthy LGG cells (L-ROS/PI^-), resulting in increased levels of H-ROS (H-ROS/PI^-, H-ROS/PI^+) and PI^+ (H-ROS/PI^+, L-ROS/PI^+, N-ROS/PI^+) populations, respectively (Figure 2-A2, B2, C2). To impose further oxidative stress, spray dried LGG cells were re-suspended in PBS and exposed to 5 mM H_2O_2 for 30 min. This rapid test intended to simulate the damage induced in bacterial powders during storage in presence of oxygen (Morgan et al., 2006). The oxidative injury caused by this treatment wiped out both H-ROS populations (H-ROS/PI^-, H-ROS/PI^+) and further increased the L-ROS/PI^+ and dead (N-ROS/PI^+) cells in dried LGG (Figure 2-B3, C3).

Based on these overall results, we speculate the following physiology for the five populations discriminated by the multiparameter method: (1) L-ROS/PI^- cells are likely to be "healthy", as they maintain ROS homeostasis and possess an intact membrane; (2) H-ROS/PI^- cells are likely to be "oxidizing", as rising levels of ROS are challenging the cell's detoxification capacity but not damaging the membrane yet; (3) H-ROS/PI^+ cells are likely to be "injured", as excess ROS are starting to oxidatively damage the membrane; (4) L-ROS/PI^+ cells are likely to be "dying", as the low levels of ROS are due to reactive depletion rather than homeostasis, and the membrane is significantly damaged; (5) N-ROS/PI^+ cells are likely to be "dead", as ROS are fully depleted and the membrane is compromised. These populations reflect the dynamic lifecycle of ROS formation, accumulation and consumption, which leads to oxidative damage of membrane macromolecules and consequent cell death (Zhao and Drlica, 2014). As the CRG probe can only detect free unreacted ROS, its stand-alone use provides a limited view of this lifecycle and leads to apparently contradictory results such as those showing disappearance of H-ROS cells and increase of L-ROS cells in dried LGG following H_2O_2 treatment (Figure 2-A2, A3).

3.2 The multiparameter FC assay reveals that H-ROS cells contribute to the fitness of LGG subjected to drying and storage stresses

The multiparameter FC assay was then used to examine the physiological changes associated with improved fitness of LGG under different drying and storage conditions.

Enhanced stability of dried probiotics during long-term storage and at challenge temperatures (above 25ºC) is a major commercial target. To achieve this, exposure to post-drying oxidative stress needs to be avoided due to the oxygen sensitivity of most probiotics (Kurtmann et al., 2009). Consequently, LGG was spray dried at an inlet temperature of either 140ºC or 170ºC and each batch of powder was stored at 37ºC either vacuum-packed (VP) or non-vacuum-packed (NVP). As expected, plate counts showed higher survival in each batch of VP powders compared to their NVP counterparts after 8 months of storage (Figure 3). Surprisingly, the multiparameter FC assay revealed that the enhanced fitness of LGG in VP powders was mainly associated with larger amounts of oxidizing (H-ROS/PI$^-$) and injured (H-ROS/PI$^+$) cells rather than of healthy cells (L-ROS/PI$^-$).

Zhang and co-authors (2010) showed that glutathione improved *L. sanfranciscensis* survival to freeze drying and freeze-thaw treatments by protecting membrane lipids from oxidation. This suggests that, despite the application of vacuum, the large temperature shift and the desiccation associated with freeze drying can trigger oxidative stress in bacteria. Consequently, we evaluated the antioxidant cysteine for its ability to protect LGG viability during an FTD challenge, which represents a rapid and reproducible lab-scale simulation of the stresses encountered during freeze drying (Date et al., 2010; Yao et al., 2009). Irrespective of the concentration of the inosine carrier, LGG tolerance to FTD was improved by the presence of cysteine as these cells grew faster in both regular and 8% NaCl-supplemented MRSC compared to those re-suspended in only inosine (Figure 4A). Faster growth in salt-supplemented medium clearly suggested a cysteine-mediated protection of LGG membrane from oxidative damage. The multiparameter FC assay confirmed this by revealing that cysteine significantly reduced the levels of dying and dead cells in favour of a larger population of injured (H-ROS/PI$^+$) cells.

The storage stability of dried bacteria can be improved by supplementing the drying medium with protective compounds and by storage at refrigeration temperatures (Morgan et al., 2006). Frozen pellets of LGG were re-suspended and freeze dried in 7 formulations (FD1-FD7) containing various combinations and amounts of proteins, sugars and other nutrients (described in section 2.2). Plate counts and the multiparameter FC assay were used to evaluate powders viability after 5 months of storage. As expected, powders stored at 4ºC

exhibited improved viability compared to those stored at 37ºC, and this was associated with the presence of larger amounts of injured and healthy cells as shown by the multiparameter FC method. In powders stored at 37ºC, the enhanced fitness of LGG in the best protective formulations was mostly associated with increased overall levels of H-ROS cells (oxidizing and injured) and, at a lesser extent, with the amounts of healthy cells (Figure 5). This was confirmed statistically as the combination of oxidizing and injured cells was found to have the highest correlation coefficient with viable plate counts, whereas healthy cells were poorly correlated (Figure 6).

These tests revealed the consistent contribution of H-ROS cells to LGG fitness under various stressful conditions. It can be inferred that these cells experience an equilibrium state between oxidative stress and lethal damage, which can be positively directed towards viability through maintenance of the intracellular redox homeostasis.

4. Conclusion

This article describes the development of a next-generation multiparameter FC method delivering a major improvement in the fluorescent analysis of oxidative stress in probiotics. By simultaneously assessing the status of the oxidative stressor (total ROS) and of the physiological target (cell membrane), this method profiles with an unprecedented depth the population heterogeneity generated by oxidation in a bacterial culture. The populations of L-ROS and H-ROS cells are not seen any more as static, progressive stages of oxidative stress accumulation as the stand-alone assay would suggest, but reveal their dynamic nature reflecting the life cycle of ROS formation, accumulation and reactive depletion. Exemplificative is the case of L-ROS cells, which is now clear can consist of healthy cells retaining ROS homeostasis and an intact membrane (L-ROS/PI^-), as well as of dying cells where ROS depletion is associated with oxidative damage of the membrane (L-ROS/PI^+). These physiologically distinct populations cannot be discriminated using stand-alone probes for oxidative stress due to their inability to detect ROS once these have reacted with a cellular target. Consequently, the multiparameter method here described ensures enhanced accuracy and represents the next-generation approach in assessing the impact of oxidative stress on bacterial fitness. The assay can be used to analyse any microorganism, not just probiotics, as both CRG and PI dyes are known to stain non-specifically any bacterium. While the data presented in this article was obtained using LGG, similar data was also generated with other probiotic strains (results not shown).

To our opinion, the significance of this study reaches far beyond method development. As any individual ROS goes through phases of formation and consumption, it is reasonable to assume that their single-parameter fluorescent analysis is affected by the same limitation observed with total ROS. This can lead to erroneous interpretation of fluorescent data and have recently misled some authors to question the involvement of ROS in antimicrobials lethality (Imlay, 2015; Keren et al., 2013), despite decades of research showing altered ROS homeostasis as mediator of antibiotic-induced bacterial death (Zhao and Drlica, 2014; Dwyer et al., 2014). Keren and co-authors (2013) used single-parameter FC and hydroxyphenyl fluorescein (HPF) to monitor the levels of individual ROS in *E. coli* upon antibiotic treatment. HPF fluorescence initially increased in cells exposed to low levels of antibiotics but gradually decreased at higher antibiotic concentrations until no difference was observed between treated and untreated cells. This apparent lack of correlation between ROS levels and cell mortality led the authors to conclude that antibiotics lethality was not associated with ROS (Keren et al., 2013). However, it seems unlikely that antibiotics would generate ROS only at low doses and not when used at higher concentrations. Based on our study's learnings around the fluorescent detection of ROS life cycle, a more plausible interpretation is that high doses of antibiotics caused large bursts of ROS, which rapidly overpowered the cell's defence systems and damaged major biomolecules. This led to reactive depletion of free ROS and cell death, which would better explain the observed decreases in HPF fluorescence and viable counts (Keren et al., 2013). Rates of ROS bursts and depletions probably increased proportionally with the intensity of the antibiotic stimulus, thereby explaining the steady decline in HPF fluorescence associated with increasing doses.

The ROS life cycle can only be detected using either a multiparameter or a real-time fluorescence approach and went unobserved by Keren and co-authors due to the technical limitations of the end-point single-parameter method. This is a broad issue as also highlighted by a recent review on the diagnosis of oxidative stress in bacteria, where the author cautioned about the interpretation of data based on ROS fluorescent probes, suggested the use of proper controls and auspicated that new measures of oxidative stress might be developed to resolve the mixed results obtained thus far (Imlay, 2015). The results of our study are in line with Imlay's remarks and suggest that mono-dimensional ROS assays generate ambiguous results because of their inability to fully depict ROS life cycle. Also, it needs to be highlighted that ROS probes (e.g. HPF, CRG) measure oxidative stress, intended as the "potential for oxidative damage to happen", whereas other probes (e.g. BODIPY 11, 2,4-Dinitrophenylhydrazine) should be used to quantify oxidative damage of biomolecules.

Clearly, the stand-alone use of these probes would still provide a limited outlook of the oxidative heterogeneity occurring in a bacterial population. This limitation can only be overcome by designing a multidimensional method where the oxidative stressor and its physiological target(s) are simultaneously assessed. The multiparameter FC method described in this paper represents an example of this next-generation approach needed to ensure reliable assessment of oxidative stress in bacteria.

5. Acknowledgements

This study was financially supported by Pfizer Consumer Healthcare (USA).

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Figure List

Figure 1. Population heterogeneity in freeze dried *Lactobacillus rhamnosus* GG (LGG) as measured by single-parameter FC analysis of oxidative stress (A) or membrane integrity (B) and by multiparameter FC analysis of oxidative stress and membrane integrity (C). Legend: N-ROS, no-ROS cells; L-ROS, low-ROS cells; H-ROS, high-ROS cells; PI^-, cells with intact membrane; PI^+, cells with damaged membrane.

Figure 2. Population heterogeneity in *Lactobacillus rhamnosus* GG (LGG) before spray drying (A1, B1, C1), after spray drying (A2, B2, C2) and in spray dried powder treated with 5 mM H_2O_2 for 30 min (A3, B3, C3) as measured by single-parameter FC analysis of oxidative stress (A) and by multiparameter FC analysis of oxidative stress and membrane integrity (B, C). Legend: N-ROS, no-ROS cells; L-ROS, low-ROS cells; H-ROS, high-ROS cells; PI^-, cells with intact membrane; PI^+, cells with damaged membrane.

Figure 3. Multiparameter FC analysis of oxidative stress and membrane integrity in *Lactobacillus rhamnosus* GG (LGG) spray dried at either 140^0C or 170^0C inlet temperature and stored either vacuum-packed (VP) or non-vacuum-packed (NVP) at 37^0C for 8 months. Cell viability levels as obtained by traditional plate counts are represented by triangle symbols and measured on the secondary vertical axis. Legend: N-ROS, no-ROS cells; L-ROS, low-ROS cells; H-ROS, high-ROS cells; PI^-, cells with intact membrane; PI^+, cells with damaged membrane.

Figure 4. Effect of cysteine on the viability of *Lactobacillus rhamnosus* GG (LGG), re-suspended in a 3 or 4% inosine carrier and subjected to freeze-thaw-desiccation (FTD) stresses, as measured by multiparameter FC analysis of oxidative stress and membrane integrity (A) and by growth curves at 37^0C in regular and 8% NaCl-supplemented MRSC medium (B). Legend: N-ROS, no-ROS cells; L-ROS, low-ROS cells; H-ROS, high-ROS cells; PI^-, cells with intact membrane; PI^+, cells with damaged membrane.

Figure 5. Multiparameter FC analysis of oxidative stress and membrane integrity in *Lactobacillus rhamnosus* GG (LGG) freeze dried in various formulations (FD1-FD7) and stored at either 37^0C or 4^0C for 5 months. Cell viability levels as obtained by traditional plate

counts are represented by triangle symbols and measured on the secondary vertical axis. Legend: N-ROS, no-ROS cells; L-ROS, low-ROS cells; H-ROS, high-ROS cells; PI^-, cells with intact membrane; PI^+, cells with damaged membrane.

Figure 6. Coefficients of correlation between cell viability, as measured by agar plate counts, and selected cell subpopulations, as identified by the multiparameter method, of *Lactobacillus rhamnosus* GG (LGG) freeze dried in various formulations (FD1-FD7) and stored at 37^0C for 5 months. Legend: L-ROS, low-ROS cells; H-ROS, high-ROS cells; PI^-, cells with intact membrane; PI^+, cells with damaged membrane.

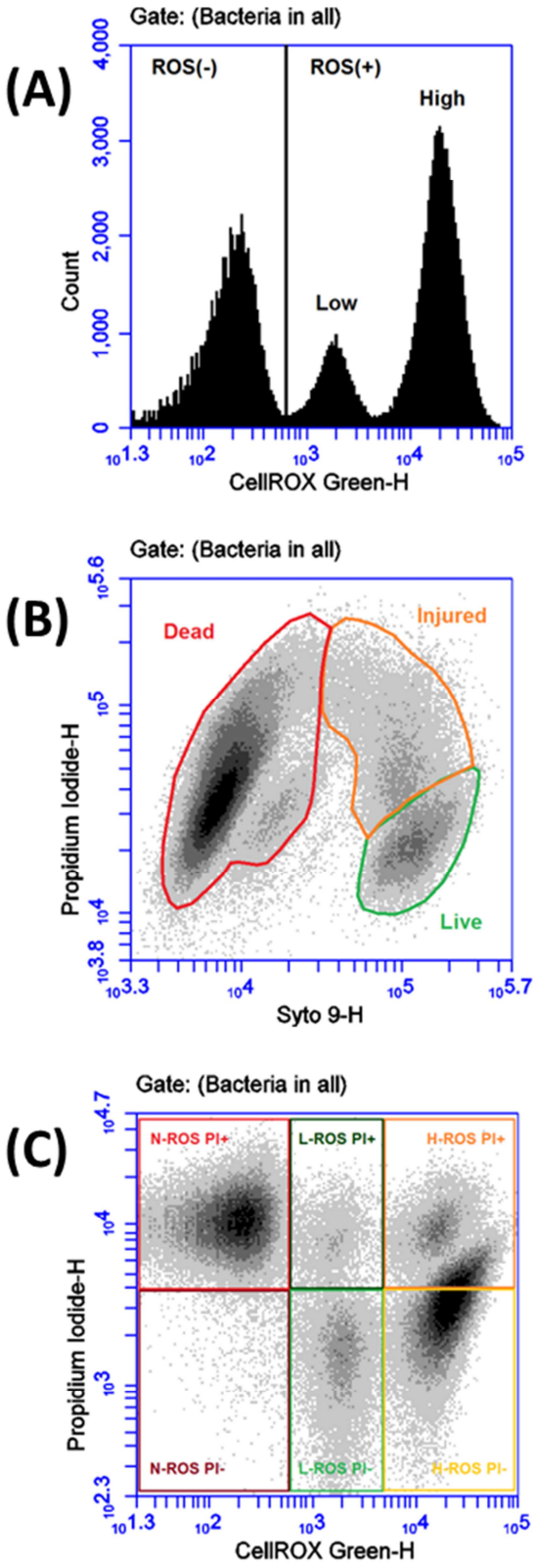


Figure 1

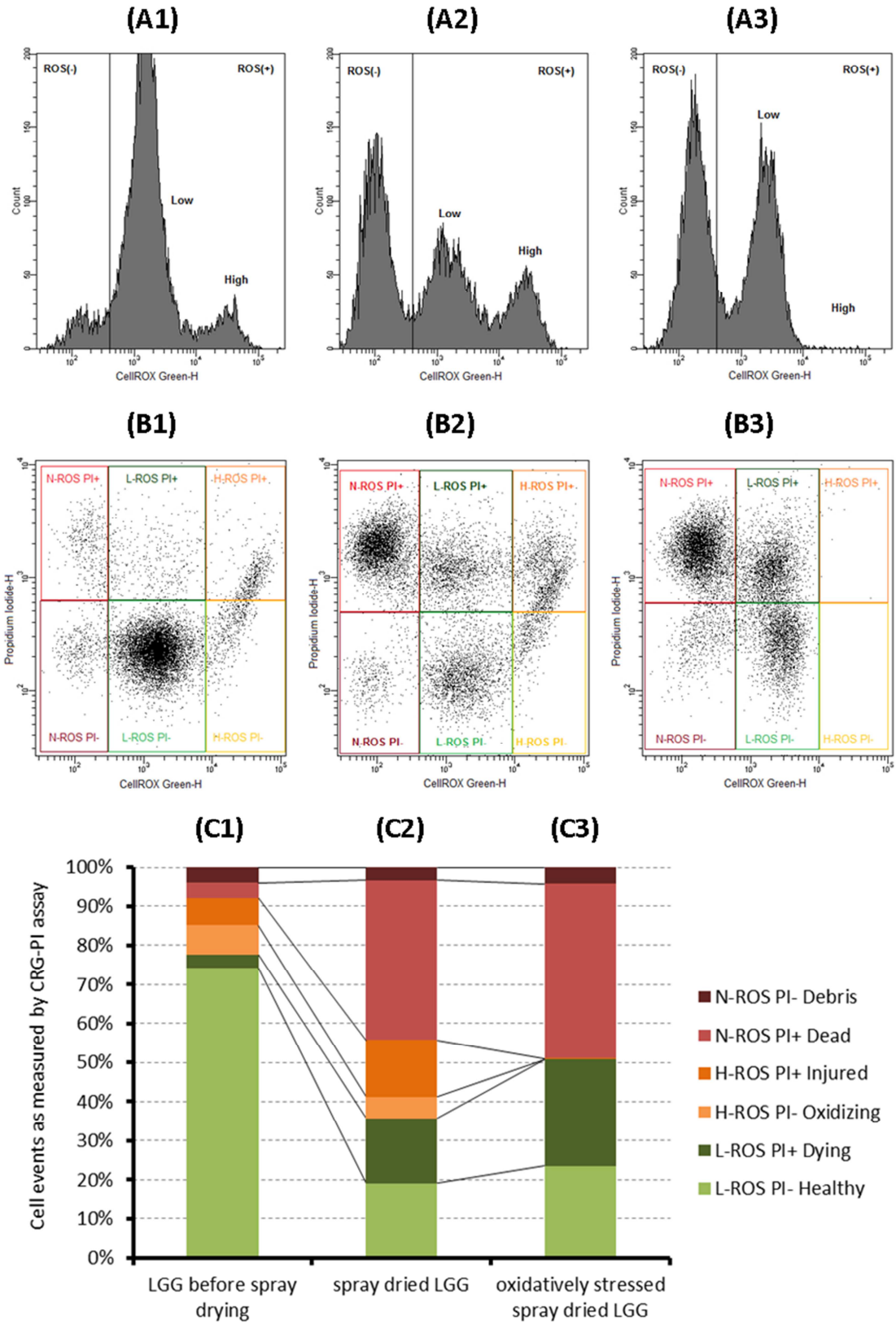
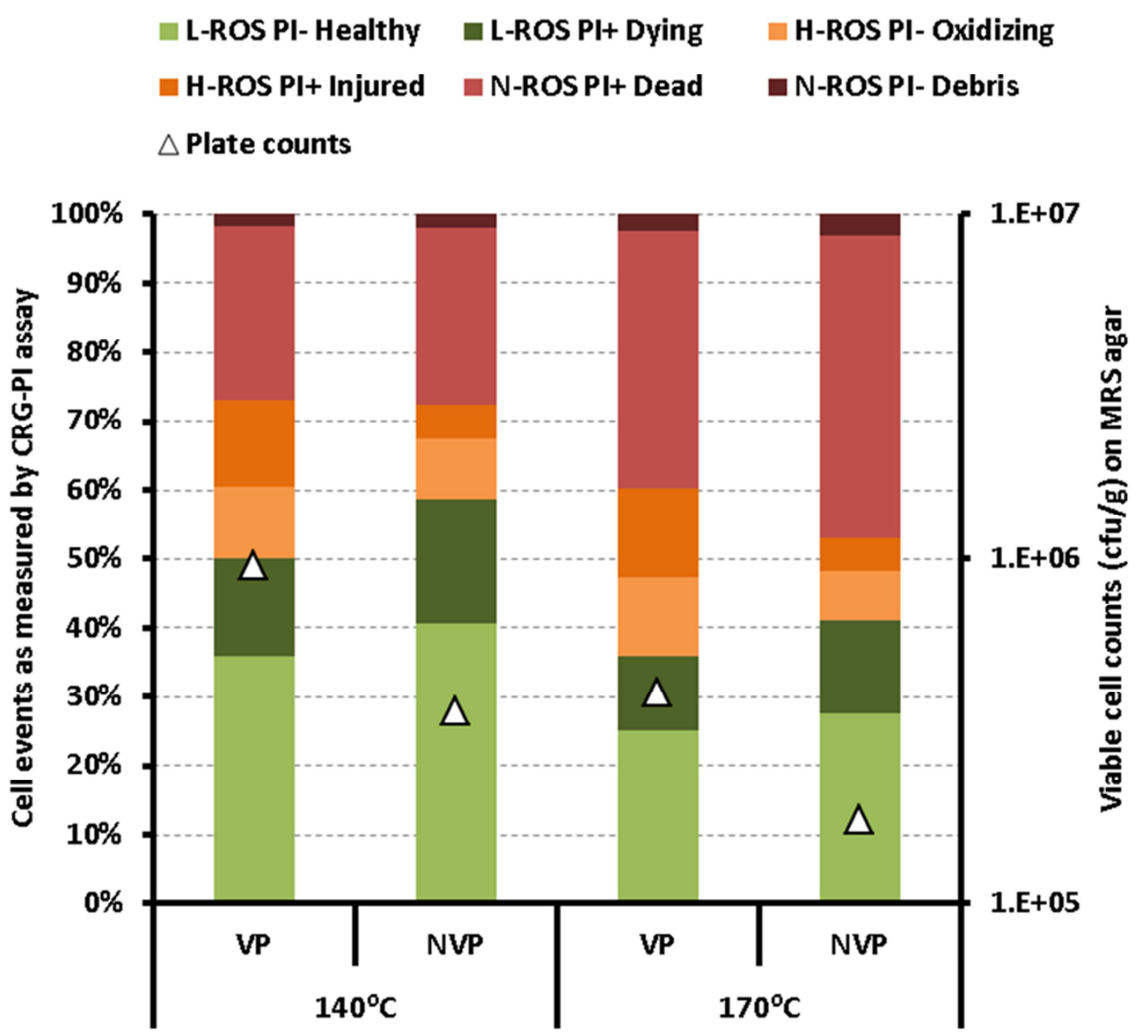

(A1)
(A2)
(A3)
ROS(-)
ROS(+)
Low
High
Count
CellROX Green-H
(B1)
(B2)
(B3)
N-ROS PI+
L-ROS PI+
H-ROS PI+
N-ROS PI-
L-ROS PI-
H-ROS PI-
Propidium Iodide-H
(C1)
(C2)
(C3)
Cell events as measured by CRG-PI assay
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
LGG before spray drying
spray dried LGG
oxidatively stressed spray dried LGG
N-ROS PI- Debris
N-ROS PI+ Dead
H-ROS PI+ Injured
H-ROS PI- Oxidizing
L-ROS PI+ Dying
L-ROS PI- Healthy

Figure 2



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535 Figure 3

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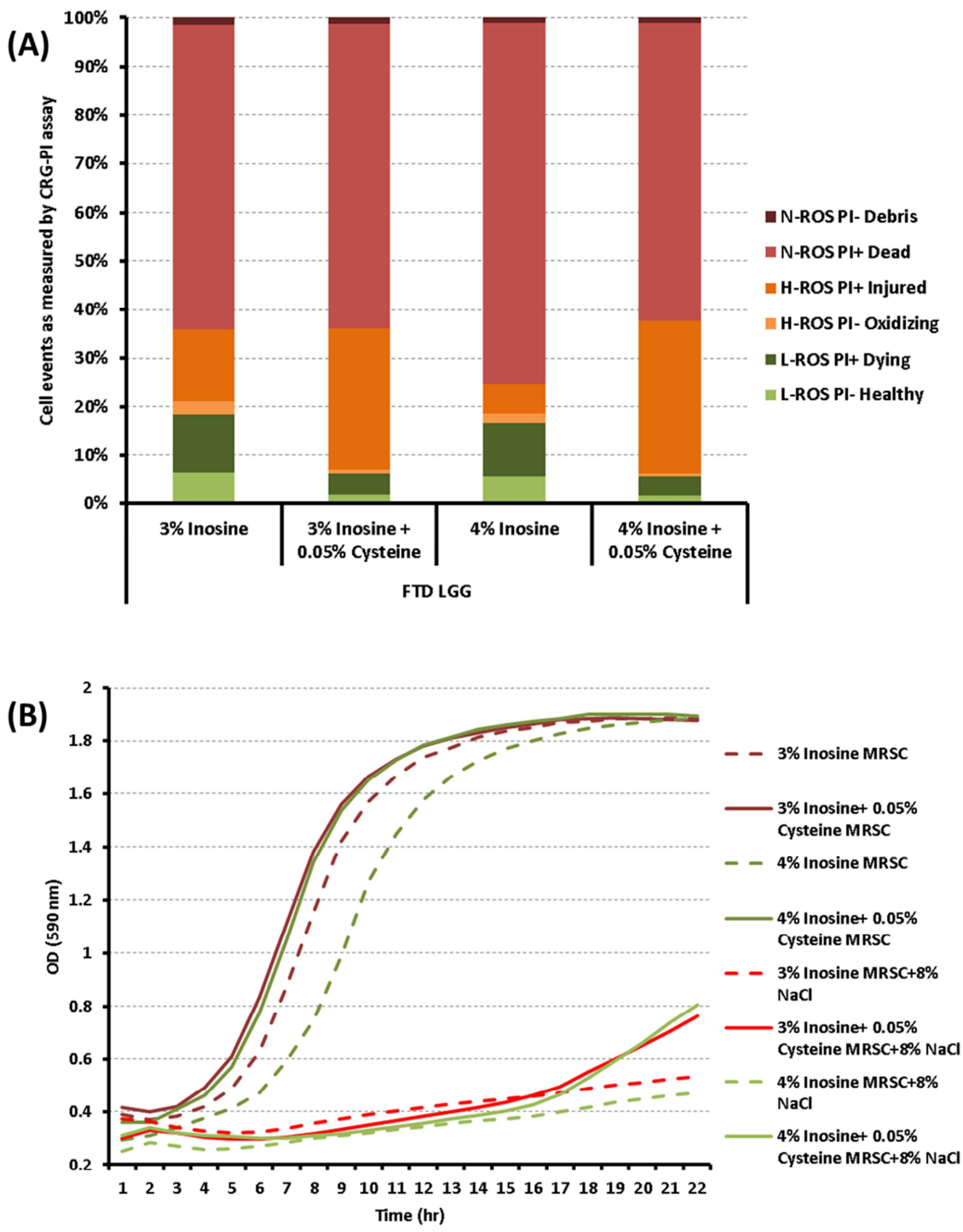

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540 Figure 4

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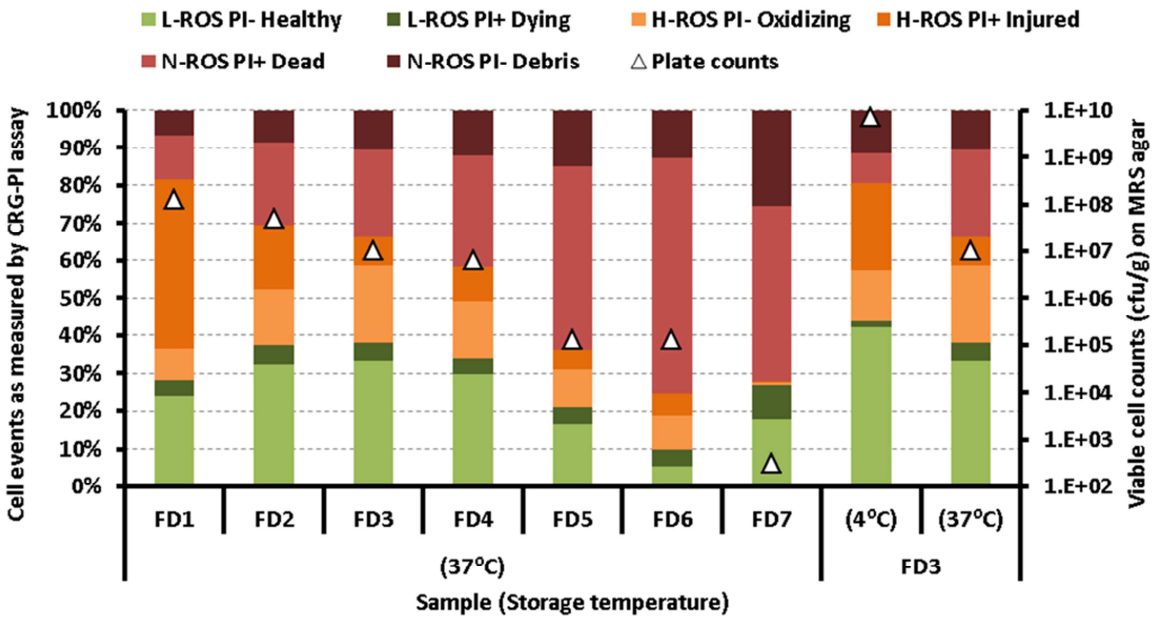

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546 Figure 5

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Cell subpopulation(s)	Correlation with Viable Plate Counts (CFU/g)
Healthy (L-ROS/PI-)	0.267
Healthy (L-ROS/PI-) + Oxidizing (H-ROS/PI-)	0.160
Healthy (L-ROS/PI-) + Oxidizing (H-ROS/PI-) + Injured (H-ROS/PI+)	0.744
Oxidizing (H-ROS/PI-) + Injured (H-ROS/PI+)	0.873

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550 Figure 6

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Manuscript Title:

Next-generation multiparameter flow cytometry assay improves the assessment of oxidative stress in probiotics

Authors, Contact information, and Affiliations:
Vincenzo Fallico[1#], Mary Rea[1], Catherine Stanton[1], Niclas Ilestam[2*], Julie McKinney[3]
[1]Teagasc Moorepark Food Research Centre, Fermoy, County Cork, Ireland
[#]**Present Address of Vincenzo Fallico**
Mail address: DuPont Nutrition & Biosciences, Madison, Wisconsin, USA
Email**: vincenzo.fallico@gmail.com**
Address of Catherine Stanton
Mail address: Teagasc, Oak Park, Carlow R93 XE12
Email: Catherine.Stanton@teagasc.ie
Address of Mary Rea
Mail address: Teagasc, Oak Park, Carlow R93 XE12
Email: Mary.Rea@teagasc.ie
[2]Pfizer Consumer Healthcare, Søborg, Gribskov, Denmark
[*]**Present Address of Niclas Ilestam**
Mail address BioGaia AB, Mobilvägen 10, SE-22362 Lund, Sweden.
Email: niclas.ilestam@biogaia.com, phone: +46702821939.

[3]Pfizer Consumer Healthcare, Richmond, Virginia, USA
Address of Julie McKinney
Mail address: 1211 Sherwood Avenue, Richmond, VA 23320
Email: jmckinney.jm52@gmail.com

Highlights

- Methods for rapid and accurate monitoring of oxidative stress in probiotics are needed as they can be instrumental to both bioprocess optimization and quality control.
- We developed a multiparameter flow cytometry method combining detection of free total reactive oxygen species with the assessment of membrane integrity.
- This allows discrimination between the stages of ROS formation, accumulation and reactive depletion, which delivers a superior profiling of the heterogeneity generated by oxidative stress in bacteria and greatly facilitates the interpretation of fluorescence-based data.

Conflict of Interest Statement

Manuscript Title:

Next-generation multiparameter flow cytometry assay improves the assessment of oxidative stress in probiotics

Authors, Contact information, and Affiliations:
Vincenzo Fallico[1#], Mary Rea[1], Catherine Stanton[1], Niclas Ilestam[2*], Julie McKinney[3]
[1]Teagasc Moorepark Food Research Centre, Fermoy, County Cork, Ireland
[#]**Present Address of Vincenzo Fallico**
Mail address: DuPont Nutrition & Biosciences, Madison, Wisconsin, USA
Email**: vincenzo.fallico@gmail.com**
Address of Catherine Stanton
Mail address: Teagasc, Oak Park, Carlow R93 XE12
Email: Catherine.Stanton@teagasc.ie
Address of Mary Rea
Mail address: Teagasc, Oak Park, Carlow R93 XE12
Email: Mary.Rea@teagasc.ie
[2]Pfizer Consumer Healthcare, Søborg, Gribskov, Denmark
[*]**Present Address of Niclas Ilestam**
Mail address BioGaia AB, Mobilvägen 10, SE-22362 Lund, Sweden.
Email: niclas.ilestam@biogaia.com, phone: +46702821939.
[3]Pfizer Consumer Healthcare, Richmond, Virginia, USA
Address of Julie McKinney
Mail address: 1211 Sherwood Avenue, Richmond, VA 23320
Email: jmckinney.jm52@gmail.com

Based on the guidance outlined in the Elsevier Conflict of Interest factsheet (https://www.elsevier.com/__data/assets/pdf_file/0007/653884/Competing-Interests-factsheet-March-2019.pdf); no conflicts of interest exist.

However, this study was financially supported by Pfizer Consumer Healthcare (USA) in partnership with Tegasc Agriculture and Food Authority (Ireland).