International Dairy Journal
February 2021, Volume 113 Pages 104889 (4p.)
https://doi.org/10.1016/j.idairyj.2020.104889
https://archimer.ifremer.fr/doc/00654/76609/

Occurrence and diversity of thermophilic sporeformers in French dairy powders

Delaunay Louis [1], Cozien Emeline [2], Gehannin Pierre [2], Mouhali Nassim [2], Mace Sabrina [2], Postollec Florence [2], Leguerinel Ivan [1], Mathot Anne-Gabrielle [1, *]

[1] Université de Brest, EA3882, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, UMT 19.03 ALTER'iX, IBSAM, 6 rue de l'Université, 29334, Quimper, France
[2] ADRIA Food Technology Institute, UMT 19.03 ALTER'iX, Z.A. de Creac'h Gwen, 29196, Quimper Cedex, France

* Corresponding author : Anne-Gabrielle Mathot, email address : mathot@univ-brest.fr

Abstract :

This study reports the prevalence of sporeforming bacteria and contamination levels of a variety of powdered dairy products from cows', ewes', goats' and mares' milk produced in France. The concentration of mesophilic spores, thermophilic spores and highly heat-resistant thermophilic spores was assessed in 61 dairy powders. Thermophilic spore concentration was highly variable between powders, likely due to the different manufacturing processes used for transforming milk into dairy powders. The different stages of processing, particularly heat treatment close to 55 °C had a strong impact on selection of thermophilic bacteria contaminating dairy powders. For some products, thermophilic spore counts were as high as 5.89 log TSC g-1. Of the 313 thermophilic isolates selected, 93.3% belonged to the species Geobacillus stearothermophilus (43.5%), Bacillus licheniformis (30.0%) and Anoxybacillus flavithermus (19.8%). These results confirm the presence of the three majority species reported worldwide.

Keywords : Spore forming bacteria, Thermophilic bacteria, dairy powders, diversity

1. Introduction

The manufacture of powdered products is an important part of the dairy industry because of their long shelf life and ease of use. France is one of the leading producers and processors of milk in Europe. Although non-pathogenic, spore-forming bacteria and more specifically thermophilic aerobic species have become an important parameter for monitoring production hygiene in this type of product. Three species dominate this group of bacteria. They include *Geobacillus stearothermophilus, Anoxybacillus flavithermus* and *Bacillus licheniformis* (Rückert, Ronimus, & Morgan, 2004).

In the dairy industry, the provenance of bacterial spores is highly diverse as spores originate from soil (Heyndrickx, 2011), water (Christiansson, Bertilsson, & Svensson, 1999) or silage (Te Giffel, Wagendorp, Herrewegh, & Driehuis, 2002). The spores can be consumed by cows, resist transit and end up in the faeces, from which they can contaminate the milk, through dirty teats and thus entering milking equipment (Miller, Kent, Boor, Martin, & Wiedmann, 2015a). Contamination can also occur during the transport of milk from the farm to the dairy industry (Huck, Sonnen, & Boor, 2008). All these sources of contamination contribute to explaining the presence of bacterial spores in raw milk (Huck, Hammond, Murphy, Woodcock, & Boor, 2007). Concentrations of mesophilic bacterial spores can be as high as 3.88 log mL^{-1} in raw milk (McGuiggan, McCleery, Hannan, & Gilmour, 2002). More specifically, thermophilic spores have been found at concentrations ranging from 0.26 to 2.41 log spore mL^{-1} in raw milk (Buehner, Anand, & Garcia, 2014; Murphy et al., 2019). Those thermophilic spores are mainly represented by *Bacillus licheniformis* but also slightly by *Geobacillus stearothermophilus* or *Anoxybacillus flavithermus* (from not detected to minus than 2%; Chauhan et al., 2013).

In addition to milk contamination and the concentration effect by processing, it has been shown that spore-forming (especially thermophilic) bacteria can multiply during processes and thereby persist in industrial sites as a biofilm (Scott, Brooks, Rakonjac, Walker, & Flint, 2007).

After pasteurisation, which has no effect on spores, the manufacturing processes for dairy powders differ according to the raw material and desired end product.

Milk powders (whole or skimmed) and whey powders are obtained by concentration in evaporators followed by atomisation, while milk protein or whey protein powders need an ultrafiltration before concentration and atomisation (see McHugh, Feehily, Hill, & Cotter, 2017 for nice scheme). In addition, other casein precipitation processes such as acid, alkali or rennet can be used. These different processes lead to different temperatures, a_w, pH conditions and durations that will select and influence the development of microorganisms. Cleaning in place operations can also affect contamination levels.

Infant formulas are another category of powder products of dairy origin, which can be manufactured either by dry formulation (mixing of powders) or wet formulation (mixing of liquid ingredients), concentrated and atomised (Happe & Gambelli, 2015).

Studies on the prevalence of mesophilic or thermophilic spore-forming bacteria have mainly focused on whole milk powders (WMPs) and skimmed milk powders (SMPs) (Dettling et al., 2019; Miller et al., 2015b; Reginensi et al., 2011; Rückert et al., 2004; Sadiq et al., 2016). However, few other studies have focused on whey powders, whey protein powders and even infant formula powders (Miller et al., 2015b; Sadiq et al., 2016; Yuan et al., 2012; Zain, Bennett, & Flint, 2017).

The objective of this study was to evaluate the contamination levels and prevalence of sporeforming bacteria in French dairy powders targeting specific products of interest for dairy powder production from cows', ewes', goats' and mares' milk.

2. Material and methods

2.1. Sampling and spore enumeration

A total of 61 French dairy powders was collected from 19 industrial plants on a voluntary base. Powders were derived from cows', ewes', goats' and mares' milk. Analysed samples were composed of WMP, SMP, whey powder (WP), whey protein concentrate (WPC), milk protein concentrate (MPC), casein powder (CP), caseinate powder (CtP), permeate powder (PP) and infant formula (IF).

Analyses were performed according to ISO 6887-5 (ISO, 2010) for milk solutions reconstitution; to NF V08-250 (NF, 2010), for mesophilic spore counts (MSC) and thermophilic spore counts (TSC), and according to ISO standard ISO/TS 27265:2009 (106 °C for 30 min; ISO, 2009) for highly heat-resistant thermophilic spore count (HRTSC).

All bacterial spores were enumerated on agar according to ISO/TS 27265 (ISO, 2009). Counts were performed in duplicate for each powder. The enumeration data were processed with Statistica version 13.5.0.17.

2.2. *Isolate recovery*

Thermophilic spore-forming bacterial counts were used to select isolates for further identification. Based on the colony morphology diversity, a proportional number of isolates were selected and isolated twice on TSA (Biokar Diagnostics) + potato starch 0.2% (Panreac). Selected isolates were stored in a 20% sterile glycerol solution at –80 °C.

2.3. *DNA extraction and isolate identification*

Procedures used for DNA extraction, M13 PCR amplification, gel migration, clustering fingerprints, isolates selection for 16S rDNA sequencing, and identification, are similar to Ronimus et al. (2003) and are fully explicated in Supplementary material.

3. Results and discussion

Milk powders analysed in this study can be classified into four major groups. This included powders processed with evaporation and atomisation (WMP; SMP and WP), which have to be re-divided because of their protein composition (into WMP and SMP on the one hand and WP on the other); powders processed with filtration step (MPC and WPC); powders made of reconstitution (IF). Finally, we arbitrarily grouped in "others" the remaining samples (CP, CtP, PP) that did not fit into these categories and for which there are few samples analysed. Enumerations and diversity between those four groups is different (Table 1).

The high concentrations of thermophilic spores in WMP or SMP (Fig. 1) from cows' milk may be explained by the temperature conditions during manufacturing processes, which were favourable for the development of thermophilic bacteria and the formation of biofilms (Zhao et al., 2013). Indeed, in plate heat exchangers used for pasteurisation, preheaters used before evaporation, and evaporators, temperatures classically range from 45 to 70 °C (Goff, 2019). In WMP and SMP, *G. stearothermophilus* seems to be predominant, followed by *B. licheniformis* and *A. flavithermus*.

International studies demonstrate that *B. licheniformis* is the main contaminant in some WMP (Rückert et al., 2004; Sadiq et al., 2016) and SMP (Sadiq et al., 2016). However, other studies demonstrate that *A. flavithermus* is also the main contaminant of some WMP (Dettling et al., 2019) and SMP (Dettling et al., 2019; Ronimus et al., 2003). Results obtained in our study are in agreement with worldwide one where *G. stearothermophilus* reaches more than 22% of identified isolates (Sadiq et al., 2016). The contamination level of WMP from ewes' and goats' milk seems less important (average: 1.68 log TSC g^{-1}) and could be linked to the size of the industry or to the run duration, in addition, no thermophilic spores were quantified in mare milk probably because of the process used (lyophilisation).

Whey can be concentrated and evaporated directly, or fractioned by filtration, giving different types of dairy powders, protein powders or permeate powders (Snappe, Lepoudere, &

Sredzinski, 2010). The high contamination of WP can be explained by evaporation steps like WMP and SMP, and filtration (for some of them), increasing the bacterial population rate. The spore diversity is quite similar to that for WMP and SMP, where *G. stearothermophilus* is predominant (52%), but *A. flavithermus* is more present in WP (29%), which is in parallel with the study from Miller et al. (2015b) who found, in acid WP, that those two sporeformers represent 27.1% and 38%, respectively.

Concentration of thermophilic spores of proteins obtained from whey (WPC) or milk (MPC) is consistent with the work of Kent, Chauhan, Boor, Wiedmann, and Martin (2016). Bacterial spores are retained by the filtration membrane into the retentate (Chamberland, Lessard, Doyen, Labrie, & Pouliot, 2017). Besides predominance of *B. licheniformis* in this type of powder was explained by Zain et al. (2017) by the development upstream of ultrafiltration processes in tanks or plate exchangers, where whey protein and lactose concentrations and temperatures are favourable for its development.

For casein powder (CP), the high level of thermophilic bacteria is due to use of rennet. There was no modification of pH value and bacteria were concentrated in the protein part. Moreover, during casein power production, rennet casein is washed with hot water (45/60 °C) which may promote thermophilic bacteria growth. The low concentration of bacterial spores in CtP may be explained by the combination of acid and alkaline treatments as well as the absence of heat treatment in plate heat exchangers or evaporation before drying. To our knowledge, no comparison data for CP or CtP are available in the bibliography. Permeate powders originate from the liquid part obtained by ultrafiltration of milk or whey (bacteria and proteins are retained in the retentate). This observation explains the absence or very limited level of 1.18 log TSC g^{-1} in one of the four permeate powder samples analysed. No correlation between diversity and process can be done due to the lack of isolates obtained from those types of powder.

Infant powders are produced by blending various milk ingredients (SMP, lactose and WPC) from the fractionation of milk constituents to approximate the composition of human breast milk. It is, therefore, difficult to explain the variability in spore concentrations observed by the impact of the manufacturing process. This difference in composition may explain the high presence of *G. stearothermophilus*, followed by *A. flavithermus* and *B. licheniformis*. These results are not in accordance with studies of Yuan et al. (2012) that demonstrated that *A. flavithermus* is the most prevalent contaminant with more than 40.4% of isolates obtained. However, Sadiq et al. (2016) demonstrated that *B. licheniformis* was the most predominant isolate with more than 47.2%. Those two studies shown that *G. stearothermophilus* was present at a level of 21% and 17.1% respectively. The contamination level of thermophilic spores in goat and ewe formula (average: 1.72 log TSC g^{-1}) seems equal to IF from cow milk, but mesophilic concentration is higher (average: 2.51 log MSC g^{-1} versus 1.06 log MSC g^{-1} in cows' milk IF).

Regarding enumeration of TSC present in the four groups, ANOVA analysis (Table 1) can classify them in two groups (a and b) resulting in a high contamination for WMP and SMP compared with IF (group b). WP, WPC and MPC are part of groups a and b due to of their median TSC contamination.

The high concentration of HRTSC (Fig. 1; Table 1) in WMP and SMP seems to be related to the presence of *G. stearothermophilus* and *A. flavithermus*, unlike MPC and WPC that were mainly contaminated by *B. licheniformis* resulting of low amount of HRTSC.

Besides the three major species, other species also isolated from the dairy environment or ingredients such as *Aneurinibacillus thermoaerophilus, Bacillus smithii*, *Brevibacillus brevis, Bacillus thermoamylovorans*, *Bacillus coagulans* and *Brevibacillus bortelensis* (Lücking, Stoeckel, Atamer, Hinrichs, & Ehling-Schulz, 2013; Miller et al., 2015b; Ronimus, Rueckert, & Morgan, 2006; Scheldeman, Pil, Herman, De Vos, & Heyndrickx, 2005; Yuan et al., 2012) or never isolated from dairy environment such as *Bacillus ginsengihumi* and *Paenibacillus naphthalenovorans* accounted for 6.71% of all the isolates in our study.

Calculated Simpson Index (1/D) (Simpson, 1949) on powder type did not highlight any difference between them, but the strain diversity reflected by the M13 PCR footprint shows that diversity of *A. flavithermus* (1/D = 4.17) is higher (Table 1) than *G. stearothermophilus* (1/D = 2.22) and *B. licheniformis* (1/D = 1.72).

4. Conclusion

The presence and concentration of mesophilic and thermophilic spore-forming bacteria in dairy powders varies according to the type of powder analysed. At each stage of milk or whey processing, the environmental conditions such as temperature, pH or composition, may or may not promote their proliferation, biofilm formation and sporulation in the raw material or residual biofilms. Furthermore, concentration processes, including evaporation, filtration or precipitation, influence the concentrations of bacterial spores in dairy powders. Thermophilic spore forming bacteria are used as a hygiene indicator if the cleaning in place procedure is not well respected. Due to their resistance to processes and cleaning products, it is important to monitor these thermophilic spores to limit their proliferation and potential impact in products using the reconstituted powders. Future experiments will be conducted on the phenotypic diversity of thermophilic spores, particularly their capacity to form biofilms and their resistance to different cleaning treatments.

Acknowledgements

The authors acknowledge the support provided by “Quimper Bretagne Occidentale” and the regional council of Brittany (Région Bretagne, France).

References

Buehner, K. P., Anand, S., & Garcia, A. (2014). Prevalence of thermoduric bacteria and spores on 10 Midwest dairy farms. *Journal of Dairy Science*, *97*, 6777–6784.

Chamberland, J., Lessard, M.-H., Doyen, A., Labrie, S., & Pouliot, Y. (2017). Biofouling of ultrafiltration membrane by dairy fluids: Characterization of pioneer colonizer bacteria using a DNA metabarcoding approach. *Journal of Dairy Science*, *100*, 981–990.

Chauhan, K., Dhakal, R., Seale, R. B., Deeth, H. C., Pillidge, C. J., Powell, I. B., et al. (2013). Rapid identification of dairy mesophilic and thermophilic sporeforming bacteria using DNA high resolution melt analysis of variable 16S rDNA regions. *International Journal of Food Microbiology*, *165*, 175–183.

Christiansson, A., Bertilsson, J., & Svensson, B. (1999). *Bacillus cereus* spores in raw milk: Factors affecting the contamination of milk during the grazing period. *Journal of Dairy Science*, *82*, 305–314.

Dettling, A., Doll, E., Wedel, C., Hinrichs, J., Scherer, S., & Wenning, M. (2019). Accurate quantification of thermophilic spores in dairy powders. *International Dairy Journal*, *98*, 64–71.

Goff, H. D. (2019). Evaporation and dehydration. Retrieved from https://www.uoguelph.ca/foodscience/book/export/html/1653

Happe, R. P., & Gambelli, L. (2015). *Infant formula*. In G. Talbot (Ed.), *Specialty oils and fats in food and nutrition: Properties, processing and applications* (pp. 285-315). London, UK: Woodhead Publishing.

Heyndrickx, M. (2011). The importance of endospore-forming bacteria originating from soil for contamination of industrial food processing. *Applied and Environmental Soil Science*, *2011*, Article 561975.

Huck, J. R., Hammond, B. H., Murphy, S. C., Woodcock, N. H., & Boor, K. J. (2007). Tracking spore-forming bacterial contaminants in fluid milk-processing systems. *Journal of Dairy Science*, *90*, 4872–4883.

Huck, J. R., Sonnen, M., & Boor, K. J. (2008). Tracking heat-resistant, cold-thriving fluid milk spoilage bacteria from farm to packaged product. *Journal of Dairy Science*, *91*, 1218–1228.

ISO. (2009). *Dried milk — Enumeration of the specially thermoresistant spores of thermophilic* *bacteria, Pub. L. No. 67.100.10, 10 ISO/TS 27265*. Geneva, Switzerland: International Standardisation Organisation.

ISO. (2010). *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products. ISO 6887-5*. Geneva, Switzerland: International Standardisation Organisation.

Kent, D. J., Chauhan, K., Boor, K. J., Wiedmann, M., & Martin, N. H. (2016). Spore test parameters matter: Mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method. *Journal of Dairy Science*, *99*, 5180–5191.

Lücking, G., Stoeckel, M., Atamer, Z., Hinrichs, J., & Ehling-Schulz, M. (2013). Characterization of aerobic spore-forming bacteria associated with industrial dairy processing environments and product spoilage. *International Journal of Food Microbiology*, *166*, 270–279.

McGuiggan, J. T. M., McCleery, D. R., Hannan, A., & Gilmour, A. (2002). Aerobic spore-forming bacteria in bulk raw milk: Factors influencing the numbers of psychrotrophic, mesophilic and thermophilic *Bacillus* spores. *International Journal of Dairy Technology*, *55*, 100–107.

McHugh, A. J., Feehily, C., Hill, C., & Cotter, P. D. (2017). Detection and enumeration of spore-forming bacteria in powdered dairy products. *Frontiers in Microbiology*, *8*, Article 00109.

Miller, R. A., Kent, D. J., Boor, K. J., Martin, N. H., & Wiedmann, M. (2015a). Different management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. *Journal of Dairy Science*, *98*, 4338–4351.

Miller, R. A., Kent, D. J., Watterson, M. J., Boor, K. J., Martin, N. H., & Wiedmann, M. (2015b). Spore populations among bulk tank raw milk and dairy powders are significantly different. *Journal of Dairy Science*, *98*, 8492–8504.

Murphy, S. I., Kent, D., Martin, N. H., Evanowski, R. L., Patel, K., Godden, S. M., et al. (2019). Bedding and bedding management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. *Journal of Dairy Science*, *102*, 6885–6900.

NF. (2010). *NF V08-250. Traitements thermiques préalables au dénombrement ou à la recherche des spores bactériennes*. AFNOR.

Reginensi, S. M., González, M. J., Olivera, J. A., Sosa, M., Juliano, P., & Bermúdez, J. (2011). RAPD-based screening for spore-forming bacterial populations in Uruguayan commercial powdered milk. *International Journal of Food Microbiology*, *148*, 36–41.

Ronimus, R. S., Parker, L. E., Turner, N., Poudel, S., Rückert, A., & Morgan, H. W. (2003). A RAPD-based comparison of thermophilic bacilli from milk powders. *International Journal of Food Microbiology*, *85*, 45–61.

Ronimus, R. S., Rueckert, A., & Morgan, H. W. (2006). Survival of thermophilic spore-forming bacteria in a 90+ year old milk powder from Ernest Shackelton's Cape Royds Hut in Antarctica. *Journal of Dairy Research*, *73*, 235–243.

Rückert, A., Ronimus, R. S., & Morgan, H. W. (2004). A RAPD-based survey of thermophilic bacilli in milk powders from different countries. *International Journal of Food Microbiology*, *96*, 263–272.

Sadiq, F. A., Li, Y., Liu, T. J., Flint, S., Zhang, G., & He, G. Q. (2016). A RAPD based study revealing a previously unreported wide range of mesophilic and thermophilic spore formers associated with milk powders in China. *International Journal of Food Microbiology*, *217*, 200–208.

Scheldeman, P., Pil, A., Herman, L., De Vos, P., & Heyndrickx, M. (2005). Incidence and diversity of potentially highly heat-resistant spores isolated at dairy farms. *Applied and Environmental Microbiology*, *71*, 1480–1494.

Scott, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M. R., & Flint, S. H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *International Journal of Dairy Technology*, *60*, 109–117.

Simpson, E. H. (1949). Measurement of diversity. *Nature*, *163*, 688–688.

Snappe, J., Lepoudere, A., & Sredzinski, N. (2010). Protéines laitières. Retrieved from: https://www.techniques-ingenieur.fr/base-documentaire/procedes-chimie-bio-agro-th2/additifs-et-adjuvants-alimentaires-42426210/proteines-laitieres-f4820/

Te Giffel, M. C., Wagendorp, A., Herrewegh, A., & Driehuis, F. (2002). Bacterial spores in silage and raw milk. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *81*, 625–630.

Yuan, D. D., Liu, G. C., Ren, D. Y., Zhang, D., Zhao, L., Kan, C. P., et al. (2012). A survey on occurrence of thermophilic bacilli in commercial milk powders in China. *Food Control*, *25*, 752–757.

Zain, S. N. M., Bennett, R., & Flint, S. (2017). The potential source of *B. licheniformis* contamination during whey protein concentrate 80 manufacture. *Journal of Food Science*, *82*, 751–756.

Zhao, Y., Caspers, M. P. M., Metselaar, K. I., De Boer, P., Roeselers, G., Moezelaar, R., et al. (2013). Abiotic and microbiotic factors controlling biofilm formation by thermophilic sporeformers. *Applied and Environmental Microbiology*, *79*, 5652–5660.

Table 1

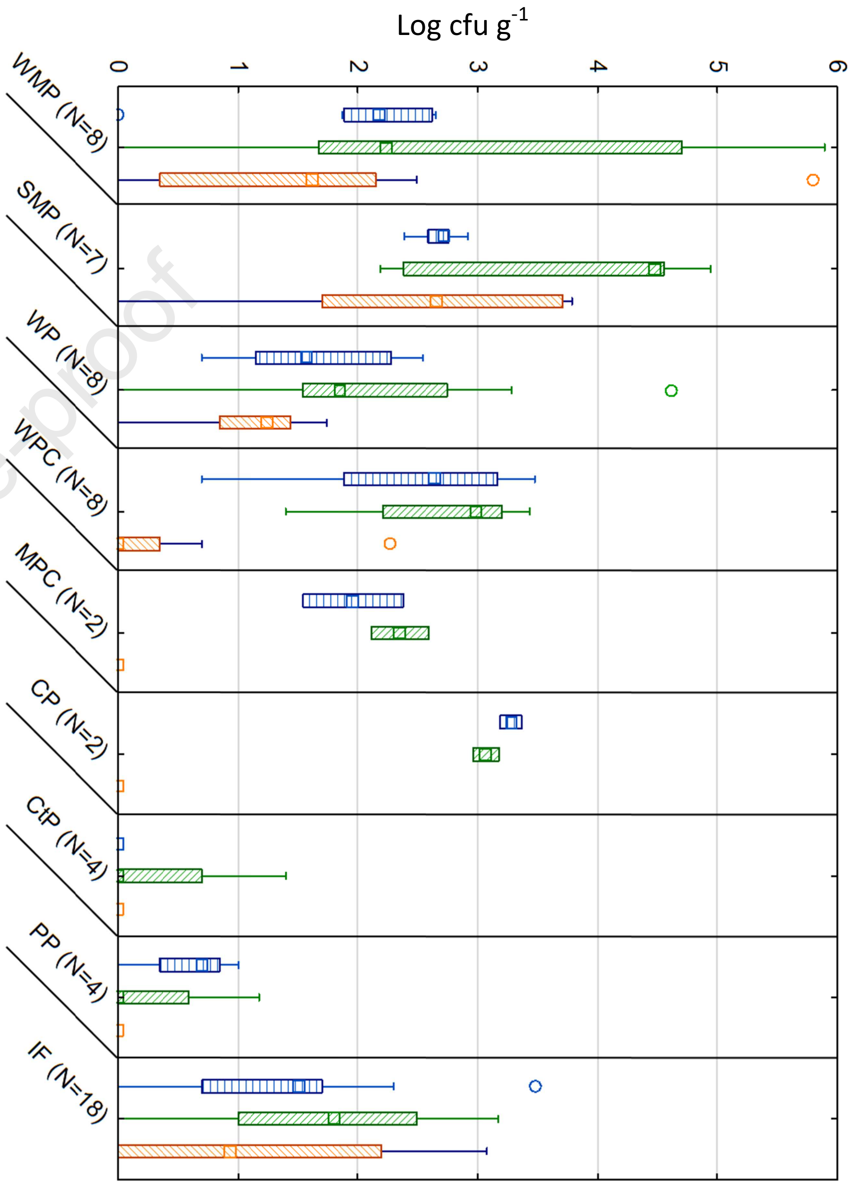
Median of enumeration of mesophilic (MSC), thermophilic (TSC) and highly heat resistant thermophilic spores (HRTSC) in the four types of dairy powders studied and diversity of TSC isolated. [a]

Parameter	WMP / SMP	WP	WPC / MPC	IF	Others	Total	Simpson index (1/D)
N samples	15	8	10	18	10	61	
Median MSC / SE	2.60 / 0.46	1.57 / 0.56	2.37 / 0.68	1.51 / 0.67	NA	NA	
Median TSC / SE	4.02 / 1.47	1.94 / 0.80	2.78 / 0.53	1.60 / 0.83	NA	NA	
Median HRTSC / SE	1.81 / 1.27	1.24 / 0.26	0 / 0.48	0.94 / 0.98	NA	NA	
ANOVA Grouping TSC	a	a/b	a/b	b	NA	NA	
Total TSC isolates	108	44	67	83	11	313	
A. flavithermus (%)	17	29	22	18	NA	19.8	4.17
B. licheniformis (%)	25	14	63	17	NA	30.0	1.72
G. stearothermophilus (%)	50	52	9	59	NA	43.5	2.22
Others (%)	8	5	6	6	NA	6.7	
Simpson index (1/D)	2.94	2.78	2.27	2.50	NA		

[a] Abbreviations are: WMP, whole milk powder; SMP, skimmed milk powder; WP, whey powder; WPC, whey protein concentrate; MPC, milk protein concentrate; IF, infant formula (Others are permeate powder, casein powder, caseinate powder); SE, standard error; NA, not applicable.

Figure legend

Fig. 1. Enumeration of mesophilic spores (▤, 80 °C 10 min, incubation 37 °C), thermophilic spores (▧, 80 °C 10 min, incubation 55 °C) and heat resistant thermophilic spores (▨, 106 °C 30 min, incubation 55 °C) in milk powders: □, median; box, 25e percentiles; whisker, non-outlier min and max (coefficient 1); ○, outlier values (coefficient 1.5). WMP, whole milk powder; SMP, skimmed milk powder; CP, casein powder; CtP, caseinate powder; MPC, milk protein concentrate; WP, whey powder; WPC, whey protein concentrate; PP, permeate powder; IF, infant formula.


Log cfu g-1
0
1
2
3
4
5
6
WMP (N=8)
SMP (N=7)
WP (N=8)
WPC (N=8)
MPC (N=2)
CP (N=2)
CtP (N=4)
PP (N=4)
IF (N=18)