

# MICROBIOLOGICAL QUALITY OF GLUTEN-FREE MEALS, NATURALLY GLUTEN FREE FOODS, AND GLUTEN FREE-LABELLED PRODUCTS

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## ABSTRACT

**Background.** The rising prevalence of gluten-related disorders such as celiac disease explains the increased consumption of gluten-free foods (GFF). However, these foods must be safe in terms of both gluten content and contamination by pathogenic microorganisms in order to avoid food poisoning.

**Objective.** The objective of this study was to assess the microbiological quality of gluten-free meals, naturally gluten free foods, and gluten free-labelled products.

**Material and Methods.** We collected 62 GFF samples including 20 meals (M-GF), 22 naturally gluten free (N-GFF) and 20 labelled (L-GFF) products, which were investigated for microbiological contamination according to Moroccan regulations guidelines, issued by the International Organization for Standardization (ISO). The analysis consisted of the detection of *Salmonella* and *Listeria monocytogenes* in each sample, and the quantification of the microbial load of the following six micro-organisms: total aerobic mesophilic flora, total coliforms, fecal coliforms, *Staphylococcus aureus*, Sulphite-Reducing Anaerobic, and yeasts and molds.

**Results.** A total of 372 analyses were carried out, showing a microbiological contamination rate of 5.1%. This contamination concerned N-GFF in 8.3% (predominantly with yeasts and molds), and meals prepared at home in 11.7% (predominantly with *Staphylococcus aureus* and coliforms). Only one case (0.8%) of contamination was observed in products labelled gluten-free and no contamination was noticed in meals prepared in food services. *Listeria monocytogenes* and *Salmonella* were not detected in any samples of food analyzed. These results indicate a good compliance of L-GFF and M-GF prepared in food services, while unsatisfactory quality was observed in N-GFF and M-GF prepared at home.

**Conclusion.** Therefore, rigorous hygienic practices and adequate corrective measures should be considered by celiac patients, especially regarding the N-GFF and M-GF prepared at home.

**Key words:** *Gluten-free foods; Microbiological quality; Contamination; Bacteria; Yeasts and Molds*

## INTRODUCTION

Celiac disease (CD) is an autoimmune disease characterized by villous atrophy of the small intestinal mucosa, crypt hyperplasia and increased intraepithelial

lymphocytes occurring in predisposed individuals [1]. Gluten remains the main factor involved in the development of this disease [2]. The prevalence of CD is generally estimated at 0.7 to 1.4% worldwide [3]. It can be asymptomatic, latent or silent and its

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diagnosis is based on serological and biopsy tests [4]. The management of CD is based almost exclusively on gluten-free diet (GFD) [5]. This diet consists of regular consumption of naturally gluten-free foods, products labelled as “gluten-free” and/or gluten-free meals prepared at food services or at home. Good adherence of celiac patients to this diet requires the availability of gluten-free foods (GFF) with reasonable price and safe gluten content [6, 7]. In addition to the elimination of any source of gluten contamination, the safety of gluten-free foods requires the absence of pathogenic microorganisms. In fact, the latter contamination by pathogens can lead to further damage to the gut microbiota of celiac patients [8], and is mainly caused by bacteria, including *Enterobacteriaceae*, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium botulinum* and *perfringens*, *Yersinia enterocolytica*, *Campylobacter jejuni*, *Brucella*, *Shigella*, *Vibrios* and *Bacillus cereus*. In Africa, *Enterobacteriaceae*, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus* and *Listeria monocytogenes* are the most involved microorganisms in this contamination [9]. This contamination can be provoked by other microorganisms such as fungi (moulds and yeasts), viruses and parasites. Hence, the study of microbiological quality of gluten-free foods will allow assessing their safety during all processes; from the harvesting of the raw material, manufacturing, transport, storage and handling to consumption. It will also help verify the effectiveness of preventive actions such as good hygiene practices (GHP) and good manufacturing practices (GMP) as well as the hazard analysis and critical control point system (HACCP).

The sanitary quality of food is assessed according to the type of micro-organisms. The presence of a single colony of virulent pathogens such as *Salmonella*, *Yersinia*, *Brucella*, *Listeria*, *salmonella* is sufficient to make it toxic and then non-compliant. Whereas less serious pathogens (aerobic mesophilic flora, sulphite-reducing anaerobes, *Staphylococci*, faecal or soil germs and yeasts and moulds) only become toxic when the number of colonies per g or per ml exceeds a toxicity threshold (S). The counting of bacteria in foods can be done by several methods such as filtration and liquid counting, but in practice, the most commonly used method is the solid medium count. This requires inoculating the microbial sample in bulk or on the surface of an agar medium, followed by the calculation of the number of colony forming units (CFU).

Due to high prevalence of CD in Morocco [10], the consumption of gluten-free foods especially naturally gluten-free foods (N-GFF) is considerably increasing. The consumption of naturally gluten-free foods such as pseudo cereals sold in bulk, is due to the low

availability and the high prices of products labelled gluten-free (L-GFP) [11-12]. This may represent an additional risk of microbiological contamination of foods to be consumed. The objective of the present study was to investigate the microbiological quality of naturally gluten-free (N-GFF), labelled gluten-free (L-GFP) products and gluten-free meals (M-GF), prepared in food services or at home.

## MATERIAL AND METHODS

### *Food samples*

The study was carried out on 62 samples of gluten-free foods, which were divided into three groups. The first group included manufactured products labelled as follow: “gluten-free”, “without gluten”, “no gluten” and “zero gluten”. The second group corresponded to naturally gluten-free foods, which do not contain wheat, rye and barley among their ingredients. Foods with labels containing terms such as ‘made in a wheat processing plant’, ‘may contain wheat traces’, ‘wheat starch’, ‘hydrolyzed wheat proteins’, ‘malt extract’, or ‘malt extract aroma’ were excluded from the study. Each group was composed of different food categories. The “Cereals/Pseudocereals GF” and “Dried vegetables GF” constituted the gluten-free labelled products (L-GFP). The “Cereals/ Pseudocereals” and “Dry Fruits/Dried vegetables” constituted N-GFF. The third group concerned “GF Meals” sold in food services or prepared at home (table 1). The food samples were collected in adherence with rigorous hygienic procedures.

### *Preparation of food samples*

The preparation of foods consisted of diluting 25 g of the sample in 225 ml of previously sterilized buffered peptone water solution (10-1 dilution) in sterile bag. The bags were then shaken in a stomacher to ensure the dispersal of the germs. After what, decimal dilutions of samples were made from a stock solution.

### *Microbiological analysis*

The microbiological analysis (detection and/or counting) of prepared foods steps were performed according to the recommendations of the National Office of Food Safety (ONSAA) and the National Institute of Standardization (IMANOR) [13], which comply with the standards settled by the International Organization for Standardization (ISO) and the French Association for Standardization (AFNOR).

### *Enumeration of total mesophilic germs (TAM)*

The enumeration of the total aerobic mesophilic flora was carried out according to the Moroccan standard NM.08.0.121, based on ISO 4833-1 [14]. One

Table 1. Categories of meals, naturally and labelled gluten-free analyzed

|                                      | Number sample | Select examples   |
|--------------------------------------|---------------|---|
| <i>Labelled gluten-free products</i> | 20            |   |
| Cereals/pseudo-cereals               | 10            | Rice, Corn, Oat, Millet   |
| Dried vegetables                     | 10            | Peas, Beans, Lentils, Haricot, Chickpeas, Soy   |
| <i>Naturally gluten-free foods</i>   | 22            |   |
| Dry Fruits/ Dried vegetables         | 12            | Cashew, Almond, Pistachio, Peanuts, Nut, Peas, Beans, Lentils, Haricot faba, Chickpeas, Soy |
| Cereals/Pseudo-cereals               | 10            | Quinoa, Chia, Sesame, Rice, Flax seed, Corn, Oat, Rice, Corn, Ryegrass                      |
| <i>Meals gluten-free</i>             | 20            |   |
| Prepared in food services            | 10            | prepared from cereals and pseudo-cereals (Bread, Cookies and Cakes)                         |
| Prepared at home                     | 10            | prepared from cereals and pseudo-cereals (Bread, Cookies and Cakes)                         |
| Overall                              | 62            |   |

ml of the stock solution and decimal dilutions were inoculated onto the PCA (Plate Count Agar) medium. The inoculum and the agar were mixed in a circular motion. Incubation was done at 30°C for 24 to 48 hours.

#### *Enumeration of total coliforms and faecal coliforms*

The enumeration of total coliforms and faecal coliforms was carried out according to NM 08.0.115 and ISO 4832 [15]. Using a sterile pipette, 1 ml of each decimal dilution was placed twice in two empty petri dishes prepared for this purpose and identified by sample type. Then, 10 to 15 ml of crystal violet and neutral red lactose agar (VRBL) was added to each petri dish, melted and cooled at 45±1°C. The inoculated plates were shaken to allow the inoculum to mix well with the agar. One set of plates was incubated at 30°C for 24 hours and furtherly used for total coliform counts. The other set of plates was incubated at 44°C for 24 hours and then used to enumerate faecal coliforms.

#### *Search and count of sulphite-reducing anaerobes (Clostridium)*

They are performed according to NM 08.0.125 from ISO 15213 [16]. 1 ml of the stock dilution and dilutions were inoculated into tubes containing 20 ml of molten Sulfite Polymyxin Sulfadiazine (SPS) agar medium and cooled to 45±1°C. The inoculum and the culture medium were mixed, without bubbling so as not to cause oxygenation of the medium, by rotating the wrist. Another layer of SPS was added to ensure a strict anaerobic medium. The tubes were incubated at 44°C for 24 h.

#### *Staphylococcus aureus testing and counting*

They were carried out according to NM 08.0.151 from ISO 6888 [17], following three steps. Firstly, isolation was done by spreading 0.1 ml of the stock

solution on the surface of the Baird Parker medium in a homogeneous way, then incubating at 37°C for 24 to 48 hours. This was followed by enrichment, during which the black, shiny, convex colonies surrounded by a zone of clearing (suspect colonies) were inoculated into the BHI (Brain Heart Infusion) broth and incubated at 37°C for 24 hours. This was followed by confirmation, during which 0.3 ml of the broth culture is added to 0.3 ml of rabbit plasma and the tubes were incubated at 37°C for 24 hours. Coagulation of the plasma indicates the presence of *Staphylococcus aureus*.

#### *Yeast and mould counts*

It was done according to NM 08.0.123 from ISO 21527 [18]. It consists of spreading 0.1 ml of the stock solution and dilutions on the surface of Sabouraud media plates, then incubating at 37°C for 24 hours.

#### *Search for Salmonella*

It was carried out according to NM 08.0.116 from ISO 6579 [19] following four steps. First, pre-enrichment by incubating the stock solution for 18 h to 24 h at 36°C. Second, enrichment by adding 0.1 ml of the pre-enriched medium to tubes containing 10 ml of Vassiliadis Rappaport broth, followed by incubation at 42°C for 18-24 hours. This was followed by isolation by exhaustion on Hektoen medium with incubation at 37°C for 24 hours. Salmonella appear as greenish or greenish colonies with a blackish centre. Suspect isolates were plated on nutrient slant agar for identification. Finally, biochemical identification through a classical gallery is performed by lactose fermentation, glucose, H<sub>2</sub>S and gas production, ONPG (ortho-Nitrophenyl-β-galactoside) test for β-galactosidase enzyme, oxidase test, and Urea-Indole test. In parallel, the biochemical identification was confirmed by the miniaturised “Api 20E” gallery consisting of 20 microtubes containing dehydrated substrates allowing 20 biochemical tests

(enzymatic or sugar fermentations) to be performed. After inoculation of the gallery, incubation was carried out at 37°C and visual reading begins after 24 hours.

#### *Testing for Listeria monocytogenes*

It was carried out according to NM 08.0.110 from ISO 11290 [20]. The pre-enrichment step was carried out by homogenising 25 g of the feed in 225 ml of Fraser demi by the stomacher and incubating for 24 hours at 37°C. Enrichment was achieved by inoculating 0.1 ml of the pre-enriched culture into 10 ml of selective Fraser broth and incubating at 37°C for 48 hours. Isolation is performed on Oxford medium and incubated at 37°C for 48 hours. Biochemical identification of the classical gallery was carried out using the haemolysis test based on the use of horse blood agar for 24 to 48 hours at 37°C. In parallel, biochemical identification was also carried out using an Api 20E gallery composed of 10 microtubes allowing 10 enzymatic tests to be performed.

In parallel, the suspect bacteria were identified using Matrix-Assisted Laser Desorption Ionisation Time-Of-Flight (MALDI-TOF MS). This identification was based on the analysis of their constituent proteins. The identification was carried out from colonies obtained on different agar media. After depositing the colony to be identified in a thin layer, a mixture of water-acetonitrile-matrix allows the bacteria to burst and release the proteins. For bacteria with a wall that is more difficult to lyse (Gram-positive bacteria), a preliminary extraction with formic acid was done before the addition of the matrix. This technique was used mainly for *Staphylococcus aureus*, sulphite-reducing anaerobes and coliforms.

The reading and interpretation of the results were carried out according to the ministerial decree n°293-19 which sets the standards for the sanitary quality of each food category in Morocco (Decree n°293-19, 2019) fixing the NM.08.0.120 as the standard regulating the expression of the results [13]. The interpretation of the results was based on the use of three-class or two-class plan depending on the microorganism to be investigated. The latter was based on the setting of a limit *m* value above which the food is considered unacceptable (non-compliant) (*m* value > 0), and acceptable (compliant) when this *m* value is equal zero (*m* value < 0). The purpose was to confirm the presence or absence of serious pathogens (*Salmonella* and *Listeria monocytogenes*). The three-class plan was used to interpret the number of aerobic mesophilic flora (TAM), sulphite-reducing anaerobes (SRA), *staphylococci* (St), coliforms total (CT), faecal coliforms (FC), yeasts and moulds (Y&M). When the counted value (*X*) is between the lower limit (*m*) and the upper limit (*M*), foods are considered conform and acceptable but the microbiological quality is

unsatisfactory. A value below *m* ( $X < m$ ) indicates a satisfactory microbiological quality. The product is considered non-compliant (unacceptable) when the enumerated value exceeds *M* value.

#### *Statistical analysis*

Data were analyzed using SPSS Statistical Package software (SPSS version 25.0, SPSS Inc., Chicago, IL, ETATS-UNIS). The prevalence was calculated as a ratio between positive and total samples, and was given as percentage. The Chi-square test was used to assess the dependence between gluten-free food categories, and the difference was considered significant if *p* value < 0.05.

## RESULTS

In the 372 microbiological analyzes carried out on 62 gluten-free foods, the contamination rate was estimated at 5.1% (Figure 1). *Total aerobic mesophilic flora*: Regarding the number of TAM flora, none of gluten-free foods were considered microbiologically unacceptable, 77.4% were acceptable, and 22.6% of them were satisfactory.

*Total coliforms & coliforms Faecal*: Only 3.2 % and 6.4 % of gluten-free foods had a higher content of total coliforms and coliforms faecal respectively. The satisfactory quality in gluten-free foods was more noticeable regarding the number of faecal coliforms than total coliforms.

*Sulphite-reducing anaerobes*: The number of feeds with unacceptable quality did not exceed 1.6% for sulphite-reducing anaerobes. The majority of products were acceptable (83.9%), while the prevalence of satisfactory microbiological quality in investigated samples was 14.6%.

*Staphylococcus aureus*: The number of *Staphylococcus aureus* over the threshold was found in seven samples, causing 11.3% of unacceptable foods. Satisfactory quality was observed in 77.4% of samples, while 11.3% of them had an acceptable microbiological quality.

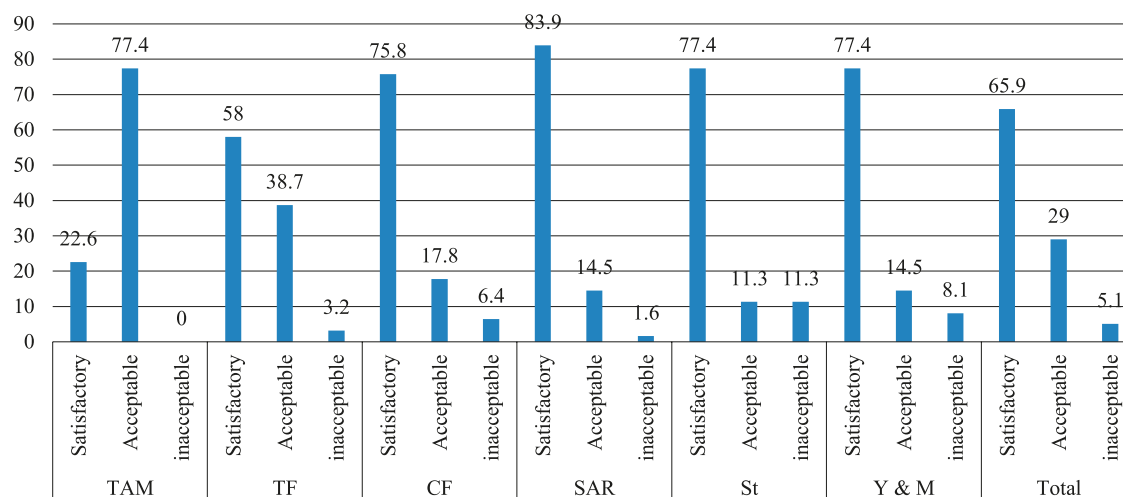
*Yeasts and moulds*: Regarding the number of yeasts and moulds, satisfactory, acceptable and unacceptable quality were noted in 77.4%, 14.5% and 8.1% respectively.

Furthermore, among the unacceptable microbiological quality foods analysed, none of them exceeded the toxic threshold ( $S=1000m$ ).

*Salmonella and Listeria monocytogenes detection*: In all gluten-free foods analysed, neither *Listeria monocytogenes* nor *Salmonella* were detected.

*Difference between L-GFP, N-GFF and meals-GF categories*

Foods labelled gluten-free were the least contaminated compared to the other categories.



Legend: TAB: Total Aerobic Bacteria; TC: Total coliforms; FC: Faecal coliforms; St: *Staphylococci Aureus*; SAR: Sulphite-Reducing Anaerobic; Y & M: Yeasts and Molds.

Figure 1: Microbiological quality of microflora counted in gluten-free foods, expressed as a percentage

Table 2. Microbiological quality of gluten-free foods according to category of products and microorganism

| Micro-organisms | Quality      | L-GFP (%) | N-GFF (%) | Meals (%)        |                          |            |
|-----------------|--------------|-----------|-----------|------------------|--------------------------|------------|
|                 |              |           |           | Prepared at home | Prepared in food service | Both-Meals |
| TAM             | Satisfactory | 35        | 9.1       | 10               | 40                       | 25         |
|                 | Acceptable   | 65        | 90.1      | 90               | 60                       | 75         |
|                 | Unacceptable | 0         | 0         | 0                | 0                        | 0          |
| TF              | Satisfactory | 80        | 50        | 40               | 50                       | 45         |
|                 | Acceptable   | 20        | 54.5      | 50               | 50                       | 50         |
|                 | Unacceptable | 0         | 4.5       | 10               | 0                        | 5          |
| FC              | Satisfactory | 90        | 77.3      | 40               | 80                       | 60         |
|                 | Acceptable   | 10        | 9.1       | 50               | 20                       | 35         |
|                 | Unacceptable | 0         | 13.6      | 10               | 0                        | 5          |
| SAR             | Satisfactory | 95        | 72.7      | 80               | 90                       | 85         |
|                 | Acceptable   | 5         | 27.3      | 10               | 10                       | 10         |
|                 | Unacceptable | 0         | 0         | 10               | 0                        | 5          |
| St              | Satisfactory | 100       | 86.4      | 30               | 60                       | 45         |
|                 | Acceptable   | 0         | 0         | 30               | 40                       | 35         |
|                 | Unacceptable | 0         | 13.6      | 30               | 0                        | 20         |
| Y & M           | Satisfactory | 85        | 50        | 100              | 100                      | 100        |
|                 | Acceptable   | 10        | 31.8      | 0                | 0                        | 0          |
|                 | Unacceptable | 5         | 18.2      | 0                | 0                        | 0          |
| Total           | Satisfactory | 80.8      | 57.6      | 50.0             | 70.0                     | 60.0       |
|                 | Acceptable   | 18.4      | 34.1      | 38.3             | 30.0                     | 34.2       |
|                 | Unacceptable | 0.8       | 8.3       | 11.7             | 0.0                      | 5.8        |

**Legend:** TAB: Total Aerobic Bacteria; TC: Total coliforms; FC: Faecal coliforms; St: *Staphylococci Aureus*; SAR: Sulphite-Reducing Anaerobic; Y & M: Yeasts and Molds; L-GFP: Labelled gluten-free products; N-GFF: Naturally gluten-free foods; M-GF : Meals gluten-free.

Satisfactory quality:  $X \leq m$ ; Acceptable quality:  $m \leq X \leq M$ ; Unacceptable quality (contaminated):  $X \geq M$  With:  $m$ : desired minimum threshold of contamination,  $M$ : maximum threshold of tolerable contamination;  $X$ =number of CFU/g in log

A Contamination by yeasts and moulds (Unacceptable quality) was noticed in 5% of L-GFP and in 18.2% of N-GFF. The latter category of foods was also contaminated by *Staphylococcus aureus*, faecal and total coliform in 13.6%, 13.6% and 5.4% of cases respectively. The gluten-free meals prepared at home were contaminated in prevalence of 11.7% mainly with *Staphylococcus aureus*. This food category was also contaminated by faecal coliforms, total coliforms and sulphite-reducing anaerobes. While, no contamination was observed in meals prepared in food services (Table 2).

## DISCUSSION

There are many aspects related to the safety of gluten-free foods such as exact gluten content and contamination by physical or chemical substances [21, 22, 23]. Microbiological contamination of foods may be responsible for intestinal food poisoning in celiac patients whose intestinal villi are already damaged by atrophy [24]. Overall, among the sample analysed in our study, the majority of gluten-free foods displayed a satisfactory microbiological quality. These results are in accordance with those reported by similar studies conducted in Italy and Brazil [25, 26]. Contamination of gluten-free meals was particularly pronounced in home-prepared meals. It was mainly caused by *Staphylococcus aureus* and coliforms, which is probably due to poor hygienic conditions. Indeed, celiac patients give great importance to the gluten content in gluten-free foods and may neglect the contamination risk. No contamination of gluten-free meals prepared in food services was observed. This may reflect the importance that restaurants and bakeries place on microbiological safety during the preparation process of these foods. An Italian study conducted in a school catering facility reported similar findings [27]. Indeed, the non-detection of a serious microbiological risk in gluten-free and lactose-free foods prepared by the services of this school confirms the compliance with good hygienic practices following HACCP implementation [27].

Contamination of products labelled as “gluten-free” was almost absent and was noticed in less than 1% of the samples. This shows that hygienic practices have been followed during all the formulation processes of gluten-free products, in accordance to the HACCP system [28].

It was remarkable that naturally gluten-free foods were frequently contaminated with yeast and mould, which could be due to poor storage conditions. This can also be explained by the fact that these foods, dedicated mainly to patients on a gluten-free diet, are generally stored for a long time before being sold. Definitely, the longer the storage period of gluten-

free foods, the more the load of yeasts and moulds increases [29]. In contrast, as a naturally gluten-free food, quinoa is generally free of microorganisms [25]. In the N-GFF of our study, coliform contamination was observed in 13.6%, which is probably related to improper handling during the processes of harvest, storage and sale.

As a serious health hazard, the presence of *Salmonella* and *Listeria monocytogenes* in gluten-free foods is alarming. In fact, *Salmonella* and *Listeria monocytogenes* are among the major causes of food-borne disease outbreaks [30]. Fortunately, no gluten-free foods have been contaminated with these dangerous bacteria. Similar findings were reported by studies conducted on L-GFP [25, 26]. Similarly, such contamination was absent in gluten-free meals prepared in food services as reported by Petruzzelli et al. [27].

At the limit of our knowledge, this study represents the first one carried out in Morocco and Africa, highlighting the importance of the microbiological safety of GFF. Our study focused on three food categories at once (N-GF, L-GFP and gluten-free meals). Nevertheless, as limitations, the sample size of foods analysed remains relatively small to draw definitive and others conclusion, especially about some more virulent food poisoning microorganisms (*Salmonella* and *Listeria Monocytogenes*). In addition, the unavailability of the *Bacillus cereus* specific agar medium in the context of this study, limited our ability to investigate the risks associated with the said organism. It is noteworthy to report, that the study *Bacillus cereus* is recommended by food regulatory organizations due to their frequent presence in foods [31].

## CONCLUSION

The results of our study showed a high prevalence of contamination in naturally gluten-free foods (8.3%) and gluten-free meals prepared at home (11.7%), predominantly with yeasts and molds for the first category, and with *Staphylococcus aureus* and coliforms for the second category of foods. While no contamination was observed in gluten-free meals prepared in food services. We also noticed the absence of contamination with some pathogens like *Salmonella* and *Listeria monocytogenes*, known for their extreme virulence. Therefore, rigorous hygienic practices and adequate corrective measures should be considered by celiac patients, especially regarding the naturally gluten-free and meals gluten-free prepared at home.

### Conflict of interest

*The authors declare that there are no conflicts of interest.*

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