Journal of Probiotics & Health

Piatti et al., J Prob Health 2016, 4:3
DOI:10.4172/2329-8901.1000158

Research Article Open Access

Old Age and Aerobic Microorganisms of Patients Affected by *Clostridium* difficile Infection are Associated Primarily with the Intestinal Presence of *Clostridium difficile*

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Abstract

Clostridium difficile infection in human occurs when the organism is present and germinating in the bowel. Old age of patients' and particular microorganisms in stools are identified as risk factors for the disease onset. We aimed to investigate if risk factors for *C. difficile* infections in a large Italian hospital were connected to *C. difficile* intestinal presence or to germination. Toxin B positivity was linked with age over 65 years (P=0.03), medical hospitalization (P=0.015) and growth of Enterobacteriaceae (P=0.029) and *Enterococcus* (P=0.05) from the same stools. The presence of *tcdB* was even more strictly linked with old age (P=0.005), medicine hospitalization (P=0.012) and growth of Enterobacteriaceae (P=0.003) and *Enterococcus* (P=0.04). Our results indicated that the presence of *C. difficile* in stools, irrespective of being spore or vegetative form, is reliably associated with old age of subjects and fecal presence of viable Enterobacteriaceae and *Enterococcus*.

Keywords: *Clostridium difficile* infection; Microbiota; Spore; Enterobacteriaceae; *Enterococcus*

Introduction

Clostridium difficile (CD) is an anaerobic, spore-forming, Gram-positive bacterium producing cytotoxic toxins, recognized in the 1970 as the cause of antibiotic-associated pseudomembranous colitis [1]. CD infection (CDI) is a major cause of hospital-acquired diarrhea. Despite the great efforts working in the ambit, infections increased in incidence and severity over the past decade and the management became more daunting, causing significant escalation of the related health-care economic burden [2]. The specific antibacterial treatment implies significant and increasing failures, recurring infections and additional modifications of microbial intestinal consortium of treated patients [3]. Prevention of CDI should be essential, but current deficit of knowledge limits the chances to advance in effective preventive measure. These limitations are due to complex CD pathogenicity, mostly concerning the health care CD acquisition and the reasons allowing the shift from bacterial intestinal presence to the infectious disease [2].

It is now ascertained that CDI is most commonly connected to increased age of patients and to changes in normal intestinal biota, the latter most due to antibiotics administration [4,5]. Several variables, individually or together, have been taken into consideration as causes of CDI onset, but little was culled as really clarifying [6,7]. The aim of our study was to analyse clinical data and intestinal presences of cultivable microorganisms to assess if the certain risk factors are connected to the pathogen staying in human bowel or to the transition from spore to vegetative form, both necessary for the onset of the disease. We evaluated age, gender, provenance of patients and microbial composition of their stools according to the presence, in the same samples, of CD in both vegetative and spore forms.

Materials and Methods

Patients and clinical specimens

From June 2011 to December 2012, unformed stools were collected from subjects affected by diarrhoea, i.e., 200 inpatients, admitted in San Martino University Hospital of Genoa, Italy, and 18 outpatients from the same regional area. All stools were sent to the Clinical Microbiology Laboratory of San Martino Hospital for detection of CD toxin B. We prospectively collected age, gender, communitarian or nosocomial provenance, and division of hospital admission. A single stool sample from each patient was included. The laboratory performed cultural, genomic, enzyme and automated immunoassay analyses of samples. The Institutional Ethics Committee of San Martino University Hospital approved the study (n. reg. CEA 13/11-Progetto ist Micro 1/2011), by dispending from informed consents.

Data collection and definitions

Diarrhoea was defined as ≥ 3 unformed stools a day during at least two consecutive days. We included in the study stools samples definitely holding CD vegetative forms, i.e., those positive for *tcdB* gene and for both CD glutamate dehydrogenase (GDH) antigen and toxin B product, discarding those CD GDH positive and toxin B negative. We enrolled, as CD GDH/toxin B negative stools, only samples which negativity was confirmed through two different analytic methods. CD spores were assumed as present in samples *tcdB* gene positive and CD GDH/toxin B negative.

Detection of CD GDH and toxin B in stools

Diarrhoeic stools were initially analysed for the presence of CD GDH and toxin B with a Rapid Membrane Enzyme Immunoassay, *C. diff* Quik Chek Complete (Techlab Inc.) by 1 hour from arrival to

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Received October 25, 2016; **Accepted** November 22, 2016; **Published** November 28, 2016

Citation: Piatti G, Mannini A, Schito AM, Musso A, Santori G (2016) Old Age and Aerobic Microorganisms of Patients Affected by *Clostridium difficile* Infection are Associated Primarily with the Intestinal Presence of *Clostridium difficile*. J Prob Health 4: 158. doi: 10.4172/2329-8901.1000158

laboratory. GDH/toxin B negative sample were analysed again with the more sensitive automated immunoassay VIDAS *C. difficile* GDH and VIDAS *C. difficile* Tox A/B [8].

Detection of *tcdB* gene in stools

Bacterial DNA was extracted from stools with the automated device QIAcube (QIAGEN). With DNA template, we performed Real-time polymerase chain reaction (RT-PCR) using *RealCycler* CDIF (Progenie Molecular), suitable to detect *tcdB* gene directly from stools, following manufacturer's instructions. Faecal presence of *tcdB* indicated presence of toxigenic CD strains, as spores and vegetative forms.

Evaluation of microbial population of stools

Stools specimens were seeded and cultured on agar plates (Columbia, MacConkey, Mannitol Salt Agar, CHROMagar Candida - BBL) in O_2 atmosphere, at 37°C for 24 hours, to value the growth of aerobic and best aerobic bacteria, including Enterobacteriaceae and Pseudomonadaceae families and *Enterococcus* and *Staphylococcus* genera, and the growth of yeasts, i.e., *Candida* genus. Colonies were identified through the growth on selective plates, microbiological tests, such as catalase, oxidase, bile-esculina agar [9] and, when needed, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Thirty stools were also collected in tubes suitable for anaerobic culture (Vacutainer Anaerobic Specimen Collector, BBL), set on blood agar plates (BBL, Bacteroides bile esculin agar) and incubated at 37°C for 48 h in anaerobiosis, to allow *Bacteroides fragilis* group and *Bacteroides fragilis* strains, strictly anaerobic bacteria, to grow. We performed PCR analysis on DNA extracted from boiled anaerobic colonies. β-isopropylmalate dehydrogenase gene (leuB) and *B. fragilis* neuraminidase gene were amplified and revealed on gel electrophoresis for detecting, respectively, the presence of *B. fragilis* group and *B. fragilis*, as described elsewhere [10,11].

Statistical analysis

Results were expressed as mean ± standard deviation, the first and third quartile (1Q, 3Q), percentage, and odds ratio (OR) with their 95% confidence intervals (CI). Continuous variables were preliminarily evaluated for normal distribution with the Shapiro-Wilk test, and then compared by the non-parametric Wilcoxon-Mann-Whitney test. Categorical variables were compared with the Pearson chi-squared test. The main variables were evaluated by recursive partitioning. Following this approach, a conditional inference tree showing both the cut-points and the P-value of the associated independent test for each node was obtained [12]. Factors associated with presence of CD toxin B or *cdtB* gene in stools specimens were evaluated by logistic regression. The independent variables that reached statistical significance at univariate analysis were entered into multivariate regression models. Statistical significance was assumed with p-values <0.05 in two-tailed tests. Statistical analysis was performed using the R software/environment (version 3.1.2; R Foundation for Statistical Computing, Vienna, Austria).

Results

Clinical data, detection of CD GDH/toxin B and *tcdB*, and aerobic faecal microbiota

We enrolled 218 not consecutive patients, whose stools met requirements**.** Seventy-five subjects were ≤ 65 years and 143 were over. Out of total patients, 178 were lying in internal medicine wards and 22 in different types of division (11 in surgery and 11 in intensive care wards), whereas 18 were outpatients (Table 1). Fourth-two stools were CD GDH/toxin B positive and 176 negative. Most stools carried live aerobic bacteria of the genus *Enterococcus* (72.5%) and family Enterobacteriaceae (60.5%). Yeasts of the genus *Candida* and bacteria of the genus *Staphylococcus* and family Pseudomonaceae grown, respectively, from 42.7%, 32.1% and 7.8 of total stools (Table 2). Amplification for *tcdB* gene was positive in 72 stools and negative in 146 (Table 3). Among 72 *tcdB* positive samples, 30 were negative for CD GDH/toxin B.

Clinical data and intestinal microbial growth according to CD GDH/toxin B presence

CD GDH/toxin B positive stools were significantly more frequent than those negative among patients aged over 65 years (P=0.03) and subjects hospitalized in internal medicine wards (P=0.015) (Table 1). Positive stools were more frequent than those negative also among subjects whose stools carried live Enterobacteriaceae and Pseudomonadaceae bacterial families (P=0.029, P=0.007) (Table 2). In specimens showing growth of *Enterococcus*, an almost significant frequency of CD GDH/toxin B positive specimens occurred (P=0.05), such as a prevalence, not reaching statistical significance, was among specimens showing growth of *Candida* and *Staphylococcus* genera. *Candida* colonies, present in the minor part of total specimens (42.7%), increased until the majority (52.4%) of those positive for CD GDH/

Demographic Characteristics	Total n. Stools	%	GDH/toxin B Positive Stools	%	GDH/toxin B Negative Stools	%	P#
Gender							
female	122	56	19	45.2	103	58.5	
male	96	44	23	54.8	73	41	
Age							
≤ 65 years	75	34.4	8	19	67	38	0.03
>65 years	143	65.6	34	81	109	61.9	
Age, mean ± SD (1Q, 3Q)		73.21 ± 16.63 (68.5, 86)			67.60 ± 20.11 (53, 82)		
Provenance							
medicine	178	81.7	40	95.2	138	78.4	0.015
others	40	18.3	2	4.8	38	21.6	
surgery	11						
intensive care	11						
ambulatory	18						

Table 1: Patients demographic data according to presence of *Clostridium difficile* GDH/toxin B in stools; #P value is indicated only when ≤ 0.05.

Organisms Grown in Stools	Total n. stools	%	GDH/toxin B Positive Stools	%	GDH/toxin B Negative Stools	%	P#
Enterobacteriaceae* y	132	60.5	32	76.2	100	56.8	0.029
Enterobacteriaceae* n*	86	39	10	23.8	76	43.2	
***Enterococcus* y**	158	72.5	36	85.7	122	69.3	0.05
***Enterococcus* n**	60	27.5	6	14	54	30.7	
***Candida* y**	93	42.7	22	52.4	71	40.3	
***Candida* n**	125	57.3	20	47.6	105	59.7	
***Staphylococcus* y**	70	32.1	16	38.1	54	30.7	
***Staphylococcus* n**	148	67.9	26	61.9	122	69.3	
***Pseudomonadaceae* y**	17	7.8	8	19.1	9	5.1	0.007
***Pseudomonadaceae* n**	201	92.2	34	80.9	167	94.9	

Table 2: Microbial fecal growth according to presence of *Clostridium difficile* GDH/toxin B in stools; *microbial family or genus grown from stool specimens; **microbial family or genus not grown from stool specimens; #P value is indicated only when ≤ 0.05.

Demographic Characteristics	Total n. stools	%	*tcdB* Positive Stools	%	*tcdB* Negative Stools	%	P#
Gender							
female	122	56	40	55.6	82	56.2	
male	96	44	32	44.4	64	43.8	
Age							
≤ 65 years	75	34.4	15	20.8	60	41.1	0.005
> 65 years	143	65.6	57	79.2	86	58.9	
Age, mean ± SD (1Q, 3Q)		73.29 ± 18.87 (68, 87)			66.90 ± 19.74 (52.25, 81)		0.043
Provenance							
medicine	178	81.7	66	91.7	112	76.7	0.012
others	40	18.3	6	8.3	34	23.3	
surgery	11						
intensive care	11						
ambulatory	18						

Table 3: Patients demographic data according to presence of *Clostridium difficile tcdB* gene in stools; #P value is indicated only when ≤ 0.05.

toxin B (Table 2). Neither specific bacterial species nor the number of species identified differed between positive and negative groups (data not shown).

Clinical data and intestinal microbial growth according to CD *tcdB* presence

By stratifying stool samples for presence and absence of *tcdB*, a significant difference was observed for both patients age over 65 years and mean age (P=0.005, P=0.043) (Table 3). A frequency significantly higher in patients *tcdB* positive than in negative was also found for hospitalization in internal medicine wards (P=0.012). Similar difference between *tcdB* positive and negative stools was observed for faecal presence of Enterobacteriaceae family and Enterococcus genus (P=0.003, P=0.04) (Table 4). The recursive partitioning analysis returned a conditional inference tree where in stool samples tcdB gene node (P=0.019) was associated with presence of Candida and Enterobacteriaceae in 34 patients (Figure 1). Neither specific bacterial species nor number of species differed after stratifying for presence and absence of *tcdB* gene (data not shown).

Logistic regression analysis for CD GDH/toxin B presence

In univariate logistic regression analysis performed for presence in stools of CD GDH/toxin B as dependent variable, statistical significance was found for age over 65 years (P=0.023), hospitalization in internal medicine wards (P=0.022), as well as for presence in stools of Enterobacteriaceae (P=0.024) and Pseudomonadaceae families (P=0.005), and *Enterococcus* (P=0.038). By entering these variables into a multivariate logistic regression model, only age over 65 years resulted no longer significant (Table 5). Although the multivariate model for CD GDH/toxin B presence revealed a high specificity (0.988), the sensitivity was very poor (0.095) (Figure 2A).

Logistic regression analysis for of CD *tcdB* presence

In univariate logistic regression performed for presence in stools of *tcdB* gene as dependent variable, statistical significance was observed for age over 65 years (P=0.004), hospitalization in internal medicine wards (P=0.01), as well as for presence in stools of Enterobacteriaceae (P=0.002) and *Enterococcus* (P=0.03). These variables resulted statistically significant when entered into a multivariate logistic regression model (Table 6). The multivariate model for *tcdB* gene in stools revealed a balanced sensitivity (0.5) and specificity (0.842) (Figure 2B).

Clinical data and microbial growth according to CD GDH/toxin B presence among *tcdB* positive specimens

We further compared frequency of old age, hospitalization in medical division and growth of best aerobic bacteria among the forty-two patients whose stools were positive for both *tcdB* gene and

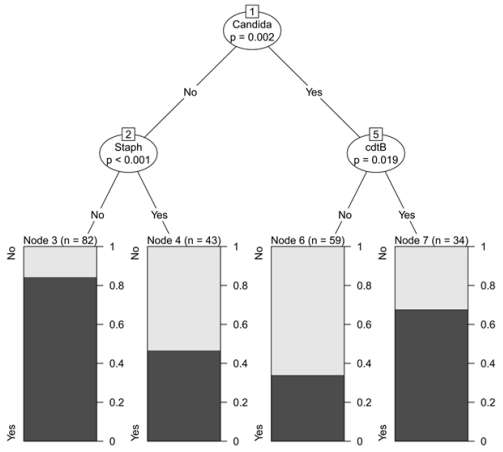

Figure 1: The best conditional inference tree returned by the recursive partitioning analysis, with both the cut-points and the p-value of the associated independent test for each node (*Candida*, *Staphylococcus*, *cdtB* gene). The columns are referred to the presence/absence (Yes/No) of Enterobacteriaceae in stool specimens.

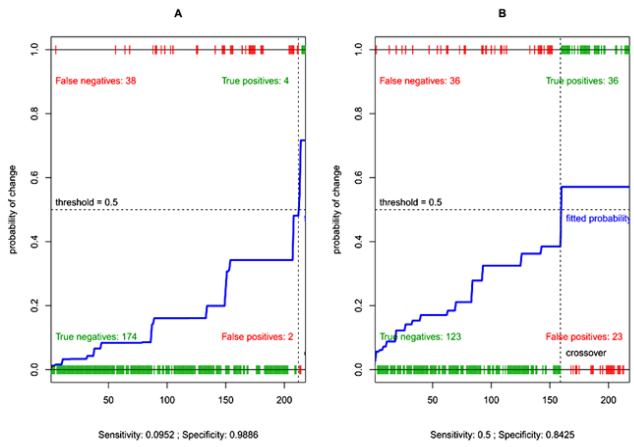

Figure 2: (A) Multivariate logistic regression model for *C. difficile* toxin B in stools (independent variables: age over 65 years; hospitalization in internal medicine wards; presence in stools of Enterobacteriaceae, *Enterococcus*, and Pseudomonadaceae). (B) Multivariate logistic regression model for the presence of *cdtB* gene in stools (independent variables: age over 65 years; hospitalization in internal medicine wards; presence in stools of Enterobacteriaceae and *Enterococcus*).

CD GDH/toxin B and among the thirty patients whose stools were positive for *tcdB* gene and negative for GDH/toxin B. The remarkable correlation found between GDH/toxin B and *tcdB* positivity and age greatly decreased, losing statistical significance. The correlation between GDH/toxin B and *tcdB* positivity and provenance of patients lost relevance. The connection with growth of best aerobic microorganisms totally disappeared, and, when we analysed thirty samples collected in tube suitable for anaerobic culture to detect viable *B. fragilis* group and *B. fragilis* strains, we did not find any difference between positive and negative groups (data not shown).

Discussion

The development of CDI in humans depends on two essential events, i.e., the pathogen presence in the gut and its germination, which lead to the production of bacterial toxins [13]. The occurrence of two different basic factors in CDI pathogenesis has made the understanding more difficult than that of most bacterial infections, and complicated over time the diagnosis and the related epidemiology [2,14]. Numerous investigations clearly demonstrated that intestinal biota, now called microbiota, has vital roles in maintaining the physiology of bowel, and that modifications, physiological or therapeutic, are fundamental in CDI onset [15,16]. Advanced age of patients is often characterized by changes of microbiota and is definitely associated with CDI [17]. We focused on a few variables that represent risk factors, to separate and quantify the relative influence they have on CD intestinal presence and on the germination process. We did not consider the exposure to antibiotics, but only some features of bowel biota, because it is ascertained that CDI onset can be independent from drug administration, though to a lesser part. In addition, it is now clear that, when CDI onset depends on antibiotics, the link is represented by modification of intestinal biota and, when not, the microbiota of affected subjects is different from that of healthy people [14].

We assessed demographic and microbiological data according to faecal presence-absence of CD GDH/toxin B and the codifying gene, *cdtB*. We found a strong connection between CD GDH/toxin B presence and age of patients over 65 years, and between the same toxin positivity and the growth of organisms of family Enterobacteriaceae and genus *Enterococcus* from the samples. These bacterial groups have emerged as frequently linked to CDI from studies conducted on mice and humans through molecular methods [4,15,16]. We found interesting, although statistically not significant, data on intestinal presence of yeasts of genus *Candida*, which, identified to a lesser extent among total stools, grew from most part of those positive for CD GDH/toxin B. The intestinal presence of CD toxin B appeared to be connected to a general presence of aerobic microbial families and genera. The lack of species specificity is reflected in previous studies where microbial modifications due to antibiotic therapies, and connected to CDI, though dissimilar among different reports, led to a same change in function [18-20]. These differences allowed the metabolic hypothesis to replace that structural, based on alleged lowering of microbiota *in toto* and on diminished competition for nutrients and ecological niches [21,22]. Given the different functional and metabolic characteristics of aerobic and anaerobic microorganisms, and a possible different role in CDI onset, we separately examined their growth. We chose culture-based methods to detect live microorganisms and to exclude those stopped in growth by any effective antibiotic therapy.

When we compared the faecal presence of GDH/toxin B with the provenance of samples, we found a strong connection between CDI and staying of patients in medical wards, which contradicts those works where surgery wards or intensive care units stand out among risk factors for the disease [23]. Interestingly, the connection of old age, faecal presence of aerobic microorganisms and medical admission with the mere presence of CD in the stools appeared more significant than the connection with the presence of GDH/toxin B. In particular, age over 65 years emerged as really determinant also in multivariate logistic regression only when we compared for *tcdB* presence. This aspect suggests that significance of old age in relation to toxin production could be misleading, i.e., due just to the presence of the microorganism in the bowel of patients.

As other authors, we thought that the changes in microbial community and in metabolic environment would allow germination

Organisms grown in stools	Total N. Stools	%	*tcdB* Positive Stools	%	*tcdB* Negative Stools	%	P#
Enterobacteriaceae* y	132	60.5	54	75	78	53.4	0.003
Enterobacteriaceae* n*	86	39	18	25	68	46.6	
***Enterococcus* y**	158	72.5	59	81.9	99	67.8	0.04
***Enterococcus* n**	60	27.5	13	18	54	32.2	
***Candida* y**	93	42.7	34	47.2	59	40.4	
***Candida* n**	125	57.3	38	52.8	87	59.6	
***Staphylococcus* y**	70	32.1	24	33.3	46	31.5	
***Staphylococcus* n**	148	67.9	48	66.7	100	68.5	
***Pseudomonadaceae* y**	17	7.8	8	11.1	9	6.2	
***Ppeudomonadaceae* n**	201	92.2	64	88.9	137	93.8	

Table 4: Microbial fecal growth according to presence of *Clostridium difficile tcdB* gene in stools; * microbial family or genus grown from stool specimens; ** microbial family or genus not grown from stool specimens; # P value is indicated only when ≤ 0.05.

	***Clostridium difficile* GDH/toxinB**	
	Univariate	**Multivariate**
	OR (95% CI) P	OR (95% CI) P
Gender, male	1.708 (0.868-3.392) 0.121	
Age	1.016 (0.997-1.037) 0.097	
Age > 65 years	2.612 (1.192-6.371) 0.023	2.095 (0.903-5.329) 0.098
Provenance, internal medicine	5.507 (1.588-34.763) 0.022	5.572 (1.516-36.475) 0.026
Presence in stools of		
Enterobacteriaceae	2.432 (1.160-5.495) 0.024	2.724 (1.228-6.528) 0.018
Enterococcus	2.655 (1.126-7.337) 0.038	2.717 (1.079-7.952) 0.046
Candida	1.626 (0.826-3.129) 0.158	
Staphylococcus	1.390 (0.679-2.780) 0.356	
Pseudomonadaceae	4.366 (1.539-12.231) 0.005	4.852 (1.571-15.235) 0.005

Table 5: Univariate and multivariate analysis of factors associated with presence of *Clostridium difficile* GDH/toxin B in stool specimens.

	***Clostridium difficile tcdB* gene**	
	Univariate	**Multivariate**
	OR (95% CI) P	OR (95% CI) P
Gender, male	1.025 (0.578-1.808) 0.932	
Age	1.015 (0.999-1.031) 0.058	
Age>65 years	2.651 (1.400-5.254) 0.004	2.342 (1.196-4.770) 0.015
Provenance, internal medicine	3.339 (1.419-9.210) 0.010	3.454 (1.423-9.761) 0.010
Presence in stools of		
Enterobacteriaceae	2.615 (1.420-4.982) 0.002	2.766 (1.453-5.445) 0.002
Enterococcus	2.154 (1.101-4.451) 0.030	2.125 (1.036-4.756) 0.045
Candida	1.319 (0.746-2.332) 0.339	
Staphylococcus	1.087 (0.590-1.974) 0.786	
Pseudomonadaceae	1.902 (0.685-5.201) 0.206	

Table 6: Univariate and multivariate analysis of factors associated with presence of *Clostridium difficile tcdB* gene in stool specimens.

from resting to vegetative morphotype of CD. To understand the causes of shift, many cross-sectional studies compared samples from patients affected by CDI with those from healthy and patients affected by non-CDI diarrhoea [24]. The lack of prospective human samples collected both before and after CDI left the underlying cause of disease unknown [16]. Here, we compared subjects as similar as possible, namely, ascertained patients carrying CD vegetative forms and patients carrying CD spores, whose diarrhoea was due to different reasons**.** In this contest, the relevance of old age, presence of best aerobic bacteria and medical admission totally disappeared. Not even the intestinal absence of *Bacteroides*, bacteria susceptible to antibiotics involved in CDI and able to inhibit the germination process appeared as favourable condition for spore germination [4,25,26]. The lack of results from the presence of *Bacteroides* could be due to a limitation of our study, i.e., the small number of stools obtained in suitable tubes. The lack of other correlations displaying the why of germination is likely due to the complexity of the subtle microbial homeostasis of human bowel. This complexity is proved by the fact that the only success in recurrent CDIs was obtained with faecal microbiota transplantation (FMT) [27]. Indeed, when FMT is efficient to recover the colonization resistance of faecal community, its composition remains different from the pre-infection status, leaving the microbiome signatures that correlate with protection unidentified [28,29].

Finally, further investigations are necessary to discover those factors that protect against germination of CD. However, the void of microbiological indications concerning the process of germination may hint interesting reconsiderations on the true meaning of data concerning the simple intestinal presence of CD. In fact, since the onset of CDI is the sum of intestinal presence and germination of CD, the less is the influence of risk factors on germination process, the greater is the influence on intestinal presence of the microorganism itself. We also suppose that the connection of CDI with admission of patients into medical wards could be due to the dietary intake of CD, instead limited in intensive care wards and in surgery divisions. Moreover, in analogy with what recently demonstrated in a murine model, where FMT has led to the clearance of CD [30], the composition of faecal microbiota of elderly patients would provide a metabolic environment favourable for durable permanence of the organism in their gut.

Conflict of Interest

The authors declare that there is no conflict of interests.

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