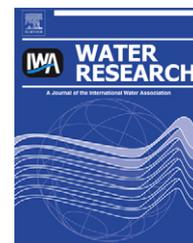


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Occurrence and diversity of *Arcobacter* spp. along the Llobregat River catchment, at sewage effluents and in a drinking water treatment plant

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ARTICLE INFO

Article history:

Received 11 January 2010

Received in revised form

18 March 2010

Accepted 6 April 2010

Available online 9 April 2010

Keywords:

Arcobacter

Water

Llobregat River

ERIC-PCR

16S rDNA-RFLP

Faecal indicators

ABSTRACT

The presence of *Arcobacter* species in faecally contaminated environmental waters has previously been studied. However, the ability to eliminate *Arcobacter* during the water treatment processes that produce drinking water has been little studied. We have investigated the prevalence and diversity of *Arcobacter* spp. throughout the year at 12 sampling points in the Llobregat River catchment (Catalonia, Spain) including 3 sites at a drinking water treatment plant. Positive samples for *Arcobacter* spp., came predominantly from the most faecally polluted sites. Recovery rates from all sites were greater in the spring (91.7%) and summer (83.3%) than in autumn and winter (75.0% in both cases), but this trend was not statistically evaluated due to the limited number of samples. Among the 339 colonies analyzed, the most prevalent species by multiplex PCR and 16S rDNA restriction fragment length polymorphism were *Arcobacter butzleri* (80.2%), followed by *Arcobacter cryaerophilus* (19.4%) and *Arcobacter skirrowii* (0.3%). Isolates showed a high genotype diversity as determined by the enterobacterial repetitive intergenic consensus PCR. In fact, 91.2% (309/339) of the colonies had different genotypes, i.e. 248 of them among the 275 isolates of *A. butzleri* and 60 among the 63 isolates of *A. cryaerophilus* and 1 genotype of *A. skirrowii*. *Arcobacter* was never detected or isolated from finished drinking water, demonstrating that water treatment is effective in removing *Arcobacter* species.

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1. Introduction

The genus *Arcobacter* was proposed by Vandamme et al. (1991) to accommodate bacteria first referred to as aerotolerant campylobacters. Currently, this genus includes nine species: *Arcobacter nitrofigilis* (the type species), *Arcobacter cryaerophilus*

(with two subgroups, 1A and 1B), *Arcobacter butzleri*, *Arcobacter skirrowii* (Vandamme et al., 1991, 1992), *Arcobacter halophilus* (Donachie et al., 2005), *Arcobacter cibarius* (Houf et al., 2005), *Arcobacter mytili* (Collado et al., 2009a), *Arcobacter thereius* (Houf et al., 2009) and *Arcobacter marinus* (Kim et al., 2010). Most of the species have been isolated from different types of

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doi:10.1016/j.watres.2010.04.002

environmental waters (Donachie et al., 2005; Collado et al., 2008, 2009a; Kim et al., 2010). The species *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* have been associated with animal and human infections (Ho et al., 2006), and it has been suggested that contaminated water can play an important role in the transmission of these microorganisms (Jacob et al., 1998; Ho et al., 2006). So far, three waterborne outbreaks have been associated with *Arcobacter* (Rice et al., 1999; Fong et al., 2007; Kopilovic et al., 2008). The first occurred in a girl school camp in Idaho, where faecal contamination was detected in the drinking water (a drilled well) that was servicing the camp (Rice et al., 1999). *Arcobacter* was also isolated in an outbreak in South Bass Island (Ohio), where a massive microbiological groundwater contamination occurred (Fong et al., 2007). More recently, *A. cryaerophilus* was isolated from a stool sample from a patient in a multi-microbial waterborne outbreak reported in Slovenia (Kopilovic et al., 2008). Although in those studies *Arcobacter* was either detected or isolated from water or from clinical sources linked to water, no genotyping studies were performed to find matches between the water and the patients' isolates.

Arcobacter have the capacity to adhere to and form biofilms in various pipe surfaces, such as stainless steel, copper, and plastic and therefore can colonize water distribution systems (Assanta et al., 2002). Despite this bacterium's susceptibility to chlorine (Rice et al., 1999; Moreno et al., 2004), Ho et al. (2006) indicated that it is still unknown if conventional treatments at a drinking water treatment plant (DWTP) can effectively remove them. In a previous study we found that *Arcobacter* spp. have a high prevalence in faecally contaminated waters (Collado et al., 2008). However, the genetic diversity of these bacteria in surface water samples has not yet been determined. Furthermore, it is also unknown whether specific genotypes are able to persist in a water source or whether the prevalence of *Arcobacter* is seasonal.

The Llobregat River (Catalonia, NE Spain) flows for 170 km from its source in the pre-Pyrenees mountains to the Mediterranean Sea, and is one of the main drinking water sources serving the metropolitan area of Barcelona. The river receives urban and industrial impacts from more than 30 sewage treatment plants (Céspedes et al., 2005). The main objective of this study was to establish the prevalence and genetic diversity of *Arcobacter* spp. in the Llobregat River catchment, including sampling sites affected by wastewater and sites at a drinking water treatment plant.

2. Materials and methods

2.1. Sampling sites

The sampling strategy covered a wide range of socio-geographical areas along the Llobregat River. The samples were taken on five occasions between February and September 2008, at the twelve sampling points (S1–S12) indicated in Fig. 1. Sampling point S1 was at the beginning of the catchment, before the river reaches the city of Berga (16,600 inhabitants), thus representing the “clean water reference site”. Sampling point S2 was at Balsareny (3300 inhabitants), where a canal transports river water to the

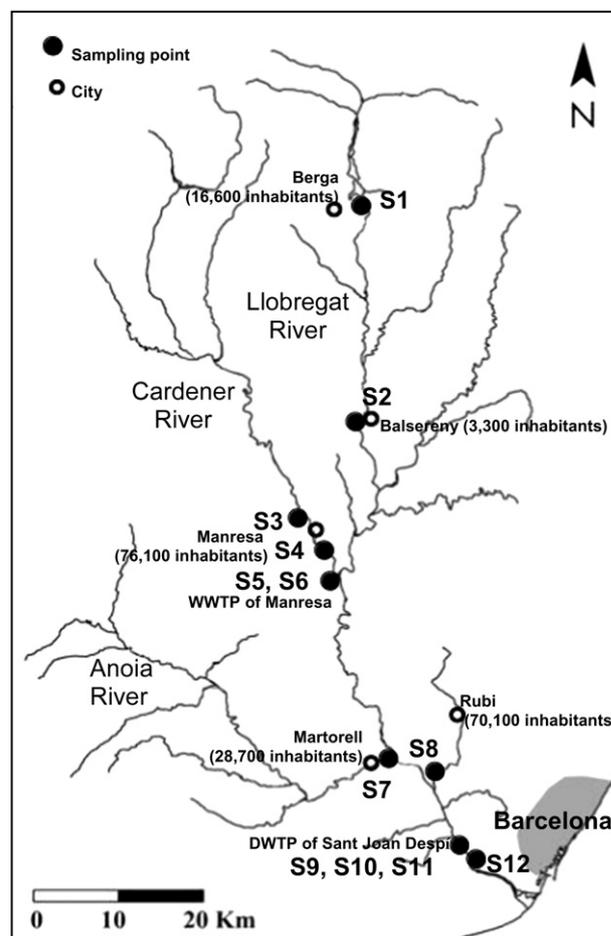


Fig. 1 – Llobregat River catchment showing sampling points S1–S12. WWTP, wastewater treatment plant; DWTP, drinking water treatment plant. Image adapted from Céspedes et al. (2005).

drinking water supply system of the city of Manresa (76,100 inhabitants). Sampling points S3 and S4 were in the Cardener River (one of the most important tributaries of the Llobregat) before and at the city of Manresa, respectively. S5 was in the inflow water of the wastewater treatment plant (WWTP) of Manresa, while S6 was the outflow of the WWTP (with secondary treatment) to the Cardener River. Sampling point S7 was at the Llobregat river water as it passes through the city of Martorell (28,700 inhabitants), where the Llobregat receives another tributary, the Anoia River. Sampling point S8 was situated in the riulet of Rubí, another tributary passing through the city of Rubí (70,100 inhabitants). Sampling point S9 was the river water where it enters the Sant Joan Despí DWTP while S10 was semi-treated drinking water, after carbon filtration and S11 corresponded to the finished drinking water after chlorination (Fig. 2). Finally, sampling point S12 was water from a canal of untreated sewage from Sant Feliu city (42,700 inhabitants) that flows into the Llobregat River below the DWTP. At each point, the water samples were collected into 2 L sterile polypropylene bottles and chilled with ice. Microbiological assays began on the same day as sampling.

Drinking Water Treatment Plant of Sant Joan Despí

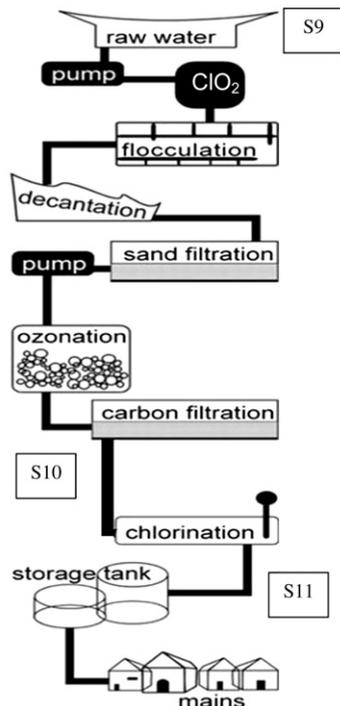


Fig. 2 – Water treatment flowchart at the Sant Joan Despí drinking water treatment plant showing sampling points S9 (raw river water), S10 (water after carbon filtration) and S11 (after chlorination).

2.2. *Arcobacter* detection and isolation procedure

For the detection and isolation of *Arcobacter* species, 200 ml of each water sample was filtered through a 0.45 µm membrane filter of 47 mm diameter (Millipore Corp., Bedford, MA, USA). The filters were rolled and placed into tubes containing 9 ml of *Arcobacter* enrichment broth (Oxoid, Basingstoke, UK) supplemented with cefoperazone, amphotericin B and teicoplanin (CAT, Oxoid) and incubated aerobically (30 °C, 48 h). After enrichment, 200 µl of the broth was transferred onto the surface of a 0.45 µm membrane filter (47 mm diameter) placed on blood agar medium and allowed to filter passively under ambient conditions for 30 min (Collado et al., 2008). Then the filters were removed and the plates were aerobically incubated (30 °C, 48–72 h). From each positive sample, eight small colourless or beige to off-white translucent colonies were picked, streaked to purity, and confirmed by Gram stain, oxidase and motility tests. Those tests identified some of the selected colonies as non-*Arcobacter* (Table 1).

2.3. *Arcobacter* molecular identification and typing

DNA was extracted from all isolates using the InstaGene™ DNA Purification Matrix (Bio-Rad Laboratories, Hercules, CA). Final identification of the presumptive isolates was made by the multiplex PCR (m-PCR) of Houf et al. (2000) and using the 16S rDNA restriction fragment length polymorphism (16S rDNA-RFLP) method of Figueras et al. (2008). The latter method

consists of an amplification of 1026 bp of the 16S rRNA gene followed by digestion with endonuclease *Mse*I that produces a species-specific pattern for all *Arcobacter* species described up to 2008, i.e. *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. nitrofigilis*, *A. cibarius* and *A. halophilus* (Figueras et al., 2008). All isolates were genotyped using the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) technique using the Houf et al. (2002) protocol. The concentration of each DNA template was determined using the GenQuant pro (Amersham Biosciences, Cambridge, England) at A_{260} and adjusted to 25 ng µl⁻¹. Gel images were saved as TIFF files, normalized with the GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, MBI Fermentas, St. LeonRot, Germany), and further analyzed by Bionumerics software, version 2.5 (Applied Maths, Kortrijk, Belgium). Patterns with one or more different bands were considered different genotypes, as in previous studies (Houf et al., 2002; De Smet et al., 2010).

2.4. Analyses of indicators of faecal pollution and viruses

The membrane filtration technique was employed for the detection of intestinal enterococci (IE) and *Clostridium perfringens* (Cp), as previously described (Figueras et al., 1996) using the ISO 7899-2 and ISO 6461-2 methods, respectively. Total coliforms (TC) and *Escherichia coli* (Ec) were analyzed using the Colilert-18 method (Quanti-Tray, IDEXX Laboratories) according to the manufacturer's instructions. All the methods are accredited by ISO 17025 standard at the AGBAR laboratory of microbiology.

Norovirus (NoV) and Hepatitis A (HAV) virus were concentrated from 10 L samples using a glass wool adsorption–elution procedure based on the method of Venter et al. (2007). Viruses in the concentrates were assayed by TaqMan real-time RT-PCR employing previously described primers (Loisy et al., 2005; da Silva et al., 2007; Pintó et al., 2009).

3. Results and discussion

3.1. Prevalence and seasonality

Arcobacter spp. were detected in 80% of the 60 samples collected, from 11 of the 12 sampling points (Table 1). All drinking water samples were negative and on only one occasion (April) the sampling point after carbon filtration (S10) was positive, with *A. butzleri* being isolated (Table 2). This species was also the only one recovered at the cleanest reference site (S1) at the beginning of the catchment (Table 2). The greatest number of samples positive for *Arcobacter* coincided with the most faecally contaminated water samples, based on the results of the bacterial faecal indicators (TC, Ec, IE and Cp) (Table 2). Greater species diversity was also found at the most contaminated sampling points (Table 2). Furthermore, all sites positive for *Arcobacter* (with the exception of S10) were coincidentally positive for the NoV analyzed by the Enteric Virus Laboratory of the University of Barcelona. In contrast, the HAV was never detected on the five sampling dates studied (U. Perez et al., manuscript in preparation). These results confirm the association of *Arcobacter* with faecally polluted

Table 1 – Prevalence and genetic diversity of *Arcobacter* species isolated from the Llobregat River catchment.

Samples			No. of identified colonies (n)		Identified species and genotypes					
Sampling month	Points (n) ^a	Positives (%)	Any <i>Arcobacter</i> spp.	Non- <i>Arcobacter</i> ^d	<i>A. butzleri</i>		<i>A. cryaerophilus</i>		<i>A. skirrowii</i>	
					No. of colonies	ERIC types	No. of colonies	ERIC types	No. of colonies	ERIC types
February	12	9 (75.0) ^a	65	7	39	33	26	26		
March	12	9 (75.0) ^a	63	9	55	49	7	7	1	1
April	12	11 (91.7) ^b	71	17	67	57	4	4		
August	12	10 (83.3) ^c	75	5	69	65	6	3		
September	12	9 (75.0) ^a	65	7	45	44	20	20		
Total	60	48 (80.0)	339	45	275	248	63	60	1	1

ERIC: enterobacterial repetitive intergenic consensus.

a S1 (Llobregat River Water at the beginning of the catchment, representing the “clean water reference site”), S10 (water after carbon filtration at the DWTP) and S11 (the finished drinking water) were negative for *Arcobacter*.

b S11 was negative for *Arcobacter*.

c S10 and S11 were negative for *Arcobacter*.

d Gram stain, oxidase and motility test identified those colonies as non-*Arcobacter*.

waters, as previously described (Collado et al., 2008). Recovery rates from all sites in our study appeared to be greater in spring (91.7%) and summer (83.3%) than in autumn and winter (75.0% in both cases). Because of the limited number of samples taken in this study no statistical analysis was performed and further studies would be required to confirm the seasonal tendency observed. Nevertheless, these results agree with the study of Stampi et al. (1999), which found that *A. butzleri* was more prevalent in sewage sludge samples during the spring/summer period in Italy, a country with very similar climatic conditions to Spain. Andersen et al. (2007) analyzed water from turkey's drinkers observing that the overall prevalence of *Arcobacter* was inversely related to the chlorination level and that there was a decline from summer 2003 (67%) when compared with the re-sampling in spring 2004 (24.7%).

Although the authors mentioned that ambient temperature may contribute to the observed seasonal variation, no winter or autumn samples were taken.

Among the 339 genetically identified *Arcobacter* isolates, *A. butzleri* was the most prevalent species (275/339, 81.1%), followed by *A. cryaerophilus* (63/339, 18.6%). All isolates of the latter species corresponded to group 1B, which is the predominant group detected in food studies (Collado et al., 2009b), but never reported before in water samples. Interestingly, as indicated previously, *A. butzleri* was the only species found in the cleaner waters. *A. skirrowii* was isolated only once in the present study (in March at S5) and this, to our knowledge, is the second report of this species being isolated from river water, the first having been in Japan (Morita et al., 2004).

Table 2 – Relationship between *Arcobacter* positive samples in the Llobregat River catchment and the mean concentrations of bacteria indicators of faecal pollution.

Sampling points ^a	<i>Arcobacter</i> positive samples (%)	<i>Arcobacter</i> isolated species (No. of genotypes)	Mean concentration of bacterial faecal indicators				Positive samples (%) for Noroviruses
			Total coliforms (MPN/100 ml)	<i>E. coli</i> (MPN/100 ml)	Intestinal enterococci (CFU/100 ml)	<i>C. perfringens</i> (CFU/100 ml)	
S1	40	Ab (2)	1.9×10^2	2.0×10^0	3.0×10^0	1.3×10^1	20
S2	100	Ab (17)–Ac (11)	4.8×10^3	8.5×10^2	3.0×10^2	6.5×10^2	80
S3	100	Ab (27)–Ac (8)	2.7×10^4	2.7×10^3	9.0×10^2	2.9×10^3	80
S4	100	Ab (24)–Ac (5)	4.0×10^4	8.6×10^3	1.7×10^3	3.2×10^3	80
S5	100	Ab (29)–Ac (6)–As (1)	2.4×10^8	1.0×10^7	5.4×10^6	2.1×10^6	100
S6	100	Ab (31)–Ac (7)	1.0×10^5	2.4×10^4	1.3×10^4	6.8×10^3	100
S7	100	Ab (21)–Ac (14)	4.3×10^4	8.3×10^3	2.2×10^3	4.4×10^3	80
S8	100	Ab (36)–Ac (1)	3.3×10^5	5.8×10^4	1.0×10^4	8.3×10^3	80
S9	100	Ab (23)–Ac (6)	1.5×10^5	1.1×10^4	2.7×10^3	1.2×10^4	80
S10	20	Ab (5)	4.0×10^0	ND	ND	1.0×10^0	0
S11	0	ND	ND	ND	ND	ND	0
S12	100	Ab (33)–Ac (2)	3.3×10^6	4.7×10^5	4.7×10^4	1.2×10^4	100

Ab, *A. butzleri*; Ac, *A. cryaerophilus*; As, *A. skirrowii*; ND, not detected.

a S1, Llobregat River Water at the beginning of the catchment, representing the “clean water reference site”; S2, Balsareny; S3, Cardener River; S4, Cardener river water at Manresa; S5, inflow water of the WWTP of Manresa; S6, outflow of the WWTP to the Cardener river; S7, Llobregat River water at Martorell; S8, at the Riera de Rubi; S9, river water entering the DWTP in Sant Joan Despí; S10, water after carbon filtration at the DWTP; S11, the finished drinking water; S12, untreated sewage which is flowing into the Llobregat River after the DWTP.

In contrast to the 80% *Arcobacter* prevalence found in the River Llobregat, only 58.6% of water samples taken in summer from other rivers in Catalonia were positive in a previous study (Collado et al., 2008). In that case, *A. butzleri* was also the dominant species (94.1%) over *A. cryaerophilus* (5.9%) (Collado et al., 2008). In a recent study in estuarine waters in southern Italy *A. cryaerophilus* and *A. butzleri* were found to be the prevailing species (Fera et al., 2010).

The 16S rDNA-RFLP and the m-PCR methods were in agreement for species identification for all strains analyzed, contrary to previous results obtained in a food survey where discordances were found between these two identification methods (Collado et al., 2009b).

The emerging human pathogens *A. butzleri* and *A. cryaerophilus* were frequently isolated from the Llobregat River and from water used to produce drinking water. Drinking water treatment effectively removed these microorganisms from the finished chlorinated water, although 1 of the 5 samples after carbon filtration was positive. *Arcobacter* are highly susceptible to chlorine (Rice et al., 1999; Moreno et al., 2004; Andersen et al., 2007), which could explain why *Arcobacter* was not detected in the finished chlorinated water. Our results agree with previous data reported by Diergaardt et al. (2004) and Aydin et al. (2007), who did not find any *Arcobacter* in drinking water samples. Jacob et al. (1998), found *A. butzleri* and other isolates defined as *A. butzleri*-like by biotyping in raw water and at treatment stages.

The sequenced genome of *A. butzleri* revealed the presence of pathways and loci often typical of environmental non-host associated organisms, as well as genes associated with virulence, suggesting that this microbe is a free-living, waterborne organism that might be classified as an emerging pathogen (Miller et al., 2007). In fact, in a recent study that employed a semi-quantitative methodology for the prioritization of foodborne zoonoses *A. butzleri* was classified as a zoonotic pathogen of significant importance (Cardoen et al., 2009).

3.2. Genotyping

ERIC-PCR was chosen to study genetic diversity because this method has successfully been applied to *Arcobacter* isolates involved in a gastrointestinal outbreak (Vandamme et al., 1993), to those recovered from poultry and beef meats (Manke et al., 1998; Houf et al., 2002; Aydin et al., 2007; De Smet et al., 2010) or from other animal faecal samples (Van Driessche et al., 2005), and for typing strains included in the descriptions of novel *Arcobacter* species (Houf et al., 2005, 2009; Collado et al., 2009a). The genetic diversity reported in those studies ranged from 11 to 58.6% for the isolates of *A. butzleri* and from 43.2 to 100% for the isolates of *A. cryaerophilus*, indicating a high genetic diversity within members of this genus. This fact, was very recently reconfirmed by the results of the first multi locus sequence typing study of the genus in which no association of sequence types (ST) with host or geographical source was found (Miller et al., 2009). However, the set of strains investigated by Miller and colleagues originated from food, animal and human samples and did not include water strains.

In our study, the 12 sampling sites along the Llobregat River not only yielded more than one species but also had multiple

ERIC-PCR genotypes (Tables 1 and 2). After eliminating redundant isolates, the prevalence of the species was 80.2% (248/309) instead of 81.1% (275/339) for *A. butzleri* and 19.4% (60/309) instead 18.6% (63/339) for *A. cryaerophilus*. In fact, 60 (95.2%) of 63 isolates of *A. cryaerophilus* were found to belong to different genotypes, as were 248 (90.2%) of the 275 isolates of *A. butzleri* (Table 1). Interestingly, in general the highest diversity of genotypes (ranging from 21 to 36 different genotypes for *A. butzleri* and from 1 to 14 for *A. cryaerophilus*) occurred in sampling sites (S3 to S9 and S12) which had higher levels of faecal indicators because they are more affected by urban sewage (Table 2). In contrast, only two and five different genotypes were found at points S1 (clean reference site) and S10 (after carbon filtration), respectively. The same genotype was never isolated from different sampling points or sampling dates, which seems to indicate that specific *Arcobacter* isolates are not able to persist or predominantly colonize the Llobregat River catchment. González et al. (2010) also never detected recurrent *Arcobacter* genotypes among the 33 investigated wastewater samples. As suggested by other authors (Aydin et al., 2007), the detection of multiple *Arcobacter* strains may indicate many sources of contamination. However, genomic rearrangement, as a consequence of multiple recombination events, has been suggested as an explanation for the high genetic diversity seen in *Campylobacter* (On, 1998) and also for *Arcobacter* (Hume et al., 2001). To our knowledge, this is the first report on the genetic diversity of *Arcobacter* isolates recovered from river and drinking water samples.

4. Conclusions

The results from the present study contribute to the assessment of the epidemiology of this emerging foodborne pathogen, showing both a high prevalence and a high genetic diversity of *Arcobacter* in faecally contaminated river water. Despite that, treatment processes used to produce drinking water were adequate for removing *Arcobacter* species from the finished water.

Acknowledgment

This work was supported by funds from the European Commission for the HEALTHY WATER project (FOOD-CT-2006-036306). The authors are solely responsible for the content of this publication. It does not represent the opinion of the European Commission. The European Commission is not responsible for any use that might be made of data appearing therein. L. C. is indebted to Universitat Rovira i Virgili for a doctoral grant. We thank Elia Bernat for the preparation of Fig. 2.

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