1 Introduction

Nowadays, Helicobacter pylori is one of the most important emerging human pathogens. Although the routes of transmission from the environment to humans are not completely defined, the entrance of H. pylori into the food chain has been identified as one of the dissemination pathways. To date, DNA of H. pylori has been found mainly in water, raw milk, meat products and fresh salads. H. pylori prevalence values range from 2-30% depending on the considered product. 

H. pylori invades the gastric mucosa and infects humans producing several digestive tract disorders, such as chronic active gastritis, peptic ulceration, and in severe cases, gastric cancer. This organism was definitively classified as the unique biological carcinogenic agent in 1994. One of the most concerning points regarding the eradication of this pathogen is the critical resistance that H. pylori has developed in recent years against the current antimicrobial applied therapies (the effectiveness has decreased to 70% compared to other antibiotic therapies against other infectious pathogens that are 95% effective) highly concerning for the scientific community. 

Under the urgent claim of the World Health Organization in 2017 to find alternative antimicrobial strategies to fight against the most resistant human pathogens, novel natural antimicrobials have been investigated. In that sense, several compounds of vegetable and animal origin have shown antimicrobial capability against H. pylori, some of which were used from ancient times to avoid gastrointestinal problems. The effect of lactoferrin from bovine milk against H. pylori was studied by Di Mario et al., (2003). This compound showed a synergistic effect against H. pylori proliferation in vivo used in combination with antibiotics. Catechins from green tea, and quercetin glycosides from apple peel, have shown strong anti-urease activity affecting H. pylori membrane functionality. Recently, propolis from the honeybee Apis mellifera has also been identified as an effective compound against this pathogen with a chemoprotective effect on gastric epithelial cells. In addition, phenolic compounds have been suggested to have a great antimicrobial potential against H. pylori. Additional studies regarding the mechanism of polyphenol action inhibiting H. pylori growth have indicated that under exposure to these antimicrobial phytochemicals, H. pylori enters in coccoid form, remaining unable to grow. Terpenes from essential oils and phenolic compounds from ginger have...
also been described as alternative natural antimicrobials against *H. pylori*,

Since the last 10 years, novel advances regarding the influence

on individual and population-based gastric cancer prevention

strategies, have been studied.\(^6\) Compounds of marine origin have recently been used as ingredients with high applications in the pharmacological, food and aesthetic industries.\(^7\)

Currently, algae constitutes a sustainable source of bioactive molecules, with brown algae from the *Phaeophyceae* group primarily rich in complex carbohydrates, with high prebiotic\(^8\) immune-modulator and antioxidant potential.\(^9,10\)

Among them, the complex fucoidan, sulphated polysaccharides of brown algae, has been described to significantly encourage the growth of beneficial intestinal microbiota, stimulates\(^11\) immune system; and inhibit the viral replication; and\(^12\) antioxidant, anti-inflammatory and anticancer properties.\(^13\) Hence fucoidan has been named as “nutrient of the future”\(^14\).

According to the review of Morya et al., (2012),\(^15\) fucoidan was first isolated in 1918, more than eight hundred articles focusing on fucoidan have been cited in PubMed.\(^16\)

Although, fucoidan extracts from algae have not been approved for use in biomedical applications, research on the bioactivity of this compound has increased exponentially in recent years (e.g. as promising agents in drug delivery, biomaterials, topical agents, and orally delivered agents for a variety of pathologies)\(^17\). Fucoidan has the status of being recognized as safe “GRAS” in the USA, Canada, Australia, and recently in Europe (approval December 2017).\(^18\) However, our knowledge only one previous publication has addressed the in vivo evaluation of fucoidan as a possible anti-*H. pylori* agent.\(^19\)

Recently, the nematode *Caenorhabditis elegans* has been incorporated as a whole animal screening platform for antimicrobials.\(^20\) This organism has a rapid generation time (300 genetically identical progeny in a 3-day life cycle).\(^21\)

Entire genome of this self-fertilizing hermaphroditic nematode has been sequenced. *Caenorhabditis elegans*, is an invertebrate animal model that has a high homology to human genome and mimics human physiological responses.\(^22,23\)

Using this novel model, Moly et al., (2009)\(^24\) tested more than 40,000 compounds and extracts, and identified 28 novel antimicrobials against *Enterococcus faecalis* and *Candida albicans*. In fact, many of the virulence factors involved in the killing of worms have been identified and also required for the pathogenesis of mammals.\(^25\)

The aim of the present study is to evaluate the in vitro and in vivo antimicrobial potential of fucoidan against *H. pylori*, by testing the effectiveness of fucoidan from three different *Phaeophyceae* species, *Fucus vesiculosus*, *Macrocystis pyrifera* and *Undaria pinnatifida*. The origin and concentrations fucoidan were evaluated in terms of bacteriostatic and bactericidal potential, and the protective effect of fucoidan against *H. pylori* proliferation was assessed in vivo using *Caenorhabditis elegans* model. The obtained results contribute to the future development of promising human therapies anti-*H. pylori*.

### Material and Methods

**Helicobacter pylori bacterial culture**

The strain of *H. pylori* used in the present study was provided by the United Kingdom Culture Type Collection with reference number 11637 NCTC. The lyophilized culture was revived according to the protocol provided by the NCTC. Cells were grown under optimal conditions (37 °C, microaerobic conditions: O\(_2\) 5 %; CO\(_2\) 15 %; N\(_2\) 85 %) in liquid Brucella Broth medium (BB) (B3051 Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 5 % (v/v) sterile foetal Bovine Serum (FBS) for 5–7 days until the stationary phase was reached.\(^37,38\) Cells recovered by centrifugation were washed three times with 5 % BB-FBS; homogenized aliquots were placed into Eppendorf tubes (2 ml) and preserved at -80 °C until use. The final concentration of the culture stock was 1x10^8 CFU/ml.

**Fucoidan extracts**

Fucoidan extracts included in the present study (purity≥95 %) were provided as powders by Merck KGaA International Company (Darmstadt, Germany) and included: *Fucus vesiculosus* (F8190 Sigma; molecular weight 82 kDa; sulfate content 24.5 % (w/w); most abundant monosaccharides: mannose (1.27 %), fucose (38 %), and galactose (3 %)); *Macrocystis pyrifera* (F8065 Sigma; molecular weight 176 kDa; sulfate content 27 % (w/w); most abundant monosaccharides: mannose (1.12 %), fucose (26 %), and galactose (4 %)); and *Undaria pinnatifida* (F8315 Sigma; molecular weight 51 kDa; sulfate content 30 % (w/w); most abundant monosaccharides: mannose (5 %), fucose (39 %), and galactose (27 %)).\(^39\)

**Fucoidan antimicrobial suspensions**

For each of the considered *Phaeophyceae* species, a stock solution at concentration of 5000 μg/mL was prepared in Müeller Hinton broth (MHB) (70192 Sigma-Aldrich; Merck KGaA International Company (Darmstadt, Germany)). For each of the independent trials, the suspensions were aliquoted in 2 ml Eppendorf tubes and stored at -20 °C for use.

Suspensions of fucoidan at concentrations of 5, 10, 25, 50 and 100 μg/mL were prepared in 10 ml tubes containing MHB+FBS (5 %) from the initial 5000 μg/mL stock. Liquid media MHB+FBS (5 % no supplemented with fucoidan) was used as the control broth.

**Inoculation and microbial analysis**

*H. pylori* stock cells were revived under optimal conditions. Stocks of bacterial solution (100 μl) was spread on plates of Columbia blood agar (CBA, Difco, Franklin Lakes, New Jersey, USA) supplemented with defibrinated horse blood (10 %) (HB, Oxoid, UK) (CBA+HB 10 %) and incubated at 37 °C under microaerobic conditions. Seven-day-old cultures were harvested by scraping the bacterial growth with a sterile swab. Recovered cells were resuspended in both (i) MHB+FBS 5 % without fucoidan (considered as a control of bacterial growth) and; (ii) MHB+FBS 5 % with [5-100] µg/ml fucoidan.
suspensions. In both cases the initial optical density (OD) at 600 nm was fitted to 0.10±0.05.

The effect of fucoidan on the microbial growth / inhibition was registered by measuring OD₆₀₀ nm.

Helicobacter pylori at 25 °C was registered by measuring OD₆₀₀ nm.

regular time intervals, 12-24h) using a Biomate 3 (Thermo Scientific, S.A.) spectrophotometer. Additionally, for each 10 cycles, 100 µl aliquots were also taken in duplicate from each plate and were considered as the total number of viable eggs laid per day by nematodes in each cohort (125 individual evaluated nematodes for each scenario).

Additionally, the H. pylori concentration (CFU per nematode) in the digestive tract of nematodes was quantified by real time quantitative polymerase chain reaction qRT-PCR assay.

Helicobacter pylori quantification by real time - quantitative polymerase chain reaction (qRT-PCR) based on SYBR green I fluorescense.

For quantification assays (concentration of H. pylori in the digestive tract during C. elegans feeding), 5 age-synchronized nematodes were placed on a plate (NGM medium or NGM+fucoidan medium); 10 plates per assay condition, and 5 repetitions per scenario were used. Nematodes were transferred every 24h to freshly prepared plate during their complete life cycle. Grown nematodes were recovered from plates at different intervals (0, 1, 3, 5, 8, 10, 12, and 15 days) and washed in drops containing 5 µl of 25 mM levamisole in M9 buffer (LM buffer) for paralysis and inhibition of pharyngeal pumping and expulsion. Then LM buffer was used to wash the nematodes twice more. Afterwards, the washed nematodes were placed in a 1.5 ml Eppendorf tube containing 50 µl of PBS buffer with 1% Triton X-100 and mechanically disrupted using a motor pestle. Nucleic acids were extracted from worm lysates using the GeneJet™ Genomic DNA Purification Kit (Fermentas, Baden-Württemberg, Germany) following the mammalian tissue protocol, according to the manufacturer’s instructions. Helicobacter DNA was detected using a LightCycler® 2.0 Instrument (Roche Applied Science, Spain) according to the optimized qRT-PCR approach developed by Pina-Pérez et al., (2018).

Statistical analysis

The significance of fucoidan antimicrobial potential against H. pylori was assessed by evaluating studied variables through ANOVA. To determine which levels of each factor were significantly different (p ≤ 0.05) a multiple range test (MRT) was applied, and the Fisher distribution (LSD) was used to check equality of variances. Statgraphics Centurion XV software (Statpoint Inc., Virginia, USA) was used for all the statistical analyses carried out in the present study.

Results

In vitro antimicrobial potential of fucoidan from Phaeophyceae species against H. pylori

Figure 1 shows the kinetic behaviour of H. pylori at 25 °C, in the control reference liquid medium MHB+FBS (5 %) and in media supplemented with fucoidan at different concentrations. As seen graphically, the highest antimicrobial effects are shown for the high fucoidan concentrations applied, independent of the fucoidan origin. Moreover, under the same incubation conditions and concentrations applied,
the origin of fucoidan significantly affected (p-value ≤0.05) 3623
bactericidal/bacteriostatic effect exerted against H. pylori 3624
Table 1 includes the fucoidan concentrations required to exert 3625
a bacteriostatic or bactericidal effect against H. pylori 3626
depending on the origin species. 327
Fucoidan [5-100] µg/ml from Undaria pinnatifida resulted in 3628
the most effective reduction in H. pylori bacterial colony 3629
(bacterial count reduction = 2 to 4 log_{10} cycles). At 3630
incubation temperature (25°C), fucoidan from Macrocystis 3631
pyridera at concentrations in the range of [50-100] µg/ml 3632
effectively inhibited bacterial growth showing bacteriostatic 3633
effects. The values of the final bacterial load remained 3634
to the initial bacterial load inoculated (±4.25±0.077 log_{10} 3635
cycles). During the first 5 days of incubation, [50-100] µg/ml 3636
fucoidan from Macrocytis pyridera effectively inhibited 3637
2 log_{10} cycles of H. pylori. Additionally, cell exposure to 3638
50 µg/ml fucoidan from Macrocytis pyridera resulted in no visible 3639
culturable (VC) forms of H. pylori after 7 days of incubation 3640
Two hypothesis can be proposed based on this result: (i) 3641
to the stressful effect that fucoidan causes on H. pylori cells 3642
the cells become in a coccoid form (viable but not culturable) 3643
VBN), or (ii) due to the bactericidal effect of the fucoidan 3644
cells become inactivated. 345
Fucoidan from Fucus vesiculosus presented a 3645
antimicrobial effect against the H. pylori population, with 3646
the lowest concentrations from [5-10] µg/ml to control bacterial 3647
growth, exerting a significant bacteriostatic effect; 2.80±0.07 3648
log_{10} cycles inhibition was observed with 10 µg/ml (7 days 3649
at 37.5°C). The antimicrobial effect of fucoidan from Fr 3650
vesiculosus was bacteriostatic at concentrations in the range 3651
[25-100] µg/ml. The higher the concentration of fucoidan 3652
faster the microbial inactivation was (p-value ≤ 0.05); [50-100] µg/ml 3653
was able to reduce the bacterial load 1.60±0.15 log_{10} 3654
2.60±0.24 log_{10} cycles, respectively, after just 24 h of micro 3655
exposure. After 3 days of exposure to fucoidan from Frvesiculosus, 3.55±0.28 log_{10} cycles of microbial inactivation 3656
were achieved in suspensions containing 100 µg/ml fucoidan. 3657
According to our results, fucoidan from brown alga Undaria 3658
pinnatifida was the most effective against H. pylori at 25°C 3659
Taking into account the exposure of bacterial cells to fucoidan 3660
after 24 h of incubation, concentration levels in the range 3661
50-100 µg/ml showed bactericidal effects. After 48 h 3662
exposure, concentrations in the range of 25-50 µg/ml achieved 3663
a reduction of 2.30±0.25 log_{10} in the VC population of H. pylori 3664
The concentration of 100 µg/ml was completely effective 3665
doing a reduction of 3.00±0.30 log_{10} in the VC population of H. pylori. 3666
The concentration of 100 µg/ml was completely effective 3667
to reducing the H. pylori VC cells to below the detection 3668
limit (bacterial effect = 4.10±0.12 log_{10} cycles). Even at 3669
higher concentrations [10-25] µg/ml, fucoidan from Undaria 3670
pinnatifida showed bactericidal effects, being able to reduce 3671
the VC population of H. pylori from 4.85±0.12 to 6.85±0.22 3672
log_{10} cycles with respect to the control, after 7 days 3673
exposure at 25 °C. 374
An ANOVA analysis was performed to determine 3195
the significance of the studied factors in reducing the level 3196
H. pylori. The fucoidan origin (p value ≤ 0.05); concentration 3197
value ≤ 0.05); and exposure time (p value ≤ 0.01) were 3198
significant factors affecting the antimicrobial capability of this 3199
bioactive compound.
For all the studied conditions, and considering the three 3200
Phaeophyceae species included in the present research, it can 3201
be concluded that the higher the exposure time of bacterial 3202
cells to fucoidan, the higher the antimicrobial effect exerted 3203
by each of the considered fucoidan suspensions. Moreover, 3204
the higher the concentration of fucoidan present in liquid media, 3205
the higher the reduction of VC counts of H. pylori, [50-100] µg/ml of fucoidan was always effective and completely 3206
reduced (>3.39-4.48 log_{10} cycles) the bacterial counts, in 2-7 3207
days depending on fucoidan origin.

Caenorhabditis elegans as a model for Helicobacter pylori infection: validation of the protective effect of fucoidan

Fucoidan from Undaria pinnatifida was selected to test the in vivo antimicrobial potential of this compound using the C. elegans model. Figure 2 shows the survival function of C. elegans fed with H. pylori in NGM medium and NGM medium supplemented with fucoidan at different concentrations. As seen graphically, there was a significant increase in the survival capability of the nematode fed with H. pylori in the presence of fucoidan, even when this compound was added at the lowest concentrations (5 µg/ml). Meanwhile, the bactericidal potential of fucoidan was exerted in vitro in the concentration range of 50-100 µg/ml, in the in vivo assay, 5 µg/ml of fucoidan from Undaria pinnatifida was effective in increasing the lifespan of C. elegans, from 10 days in NGM (C. elegans fed with H. pylori) to 17 days in NGM + 5 µg/ml fucoidan. The addition of fucoidan at a concentration 225 µg/ml increased the lifespan of the nematode (26±2 days) even more than the lifespan observed in the reference (23 days lifespan when C. elegans in NGM was fed with optimal E. coli OP50).
Regarding the fertility assay, C. elegans N2 fed with E. coli OP50 in NGM (reference conditions) showed an RS equal to 155±23 eggs laid per day in the first 7 days, with egg-laying significantly reduced from day 5 of the life cycle. Under the H. pylori feeding pattern in NGM, the nematodes egg-laying was interrupted just after the first 36 h, and in many cases, worms retained eggs in bag (36 out of 125). The number of viable offspring was also reduced significantly in nematodes fed with H. pylori in relation to nematodes fed under reference conditions (E. coli OP50) (Table 2). Working with NGM supplemented with fucoidan at different concentrations, the reproductive timing of C. elegans was extended (5-12 days) and additionally, the number of laid eggs was increased, which was directly related to the concentration of fucoidan added to the media (see Table 2).
To establish a relationship between C. elegans infection (reduced lifespan and reduced egg laying) and H. pylori accumulation in the digestive tract, H. pylori was quantified across the lifespan of the nematode by qRT-PCR. Table 3 shows the quantitative values detected for H. pylori depending on considered scenarios. C. elegans fed with H. pylori seeded in NGM medium showed an increase in the intestinal load

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from < 10^2 H. pylori CFU/worm on day 0 (L4 stage) to 434
379 CFU/worm on day 7, and only 12 % of the initial population 435
380 was able to survive. Nematodes grown on NGM+ fucoidan 436
381 plates showed < 10^2 H. pylori CFU/worm under ≥ 10 µg/ml 437
382 fucoidan exposure, with no significant differences between 438
383 digestive colonization of C. elegans when fucoidan was added 439
384 in the range 10-100 µg/ml.

385

Discussion

386 Fucoidans from Phaeophyceae have been described to 433
387 be more effective as antitumoral agents than other fucoidans 434
388 from other algae species, even more, fucoidans from 435
389 Phaeophyceae have a wide spectrum of functionalities 436
390 attributed to other fucoidans.23,24,43 The present study 437
391 concludes the significant differences between the fucoidans 438
392 from three species of Phaeophyceae evaluated for their 439
393 antimicrobial capability against H. pylori. Although 440
394 fucoidans resulted in all cases effective exerting both 441
395 bacteriostatic and bactericidal effects 44,53 Undaria pinnatifida 442
396 was the most effective, with bactericidal potential even at the 443
397 lowest concentrations [5-10] µg/ml, depending on the exposure 444
398 time. Previous studies outlined that fucoidan 445
399 ingestion, the ingestion of H. pylori infection, with the 450
400 effectiveness of the treatment optimized based on the 451
401 ingested fucoidan concentration (anti-adherence / 452
453 anti-biofilm potential) and killing effect on H. pylori, and 454
455 also based to treatment exposure time.50,51,52,54

456 Bioactive compounds from algae have also been tested in vivo 457
459 using the C. elegans model.55,56,57 During its growth, the worm 460
458 intake nutrients from the medium, in addition to the 461
459 ingestion of the bacterial food source (E. coli OP50 or H. pylori 462
460 in this case). Methanolic extracts from red alga, Chondrus 463
464 crispus, have been demonstrated to increase the C. elegans 465
466 lifespan increasing the oxidative stress tolerance of the 467
468 nematode.56 Astaxanthin (AX) from marine origin has also 469
470 been described with high impact, increasing the lifespan of C. 471
elegans populations fed on medium supplemented with 0.1 to 472
473 1 mM AX by 16-30 % (E. coli OP50 as food source).54 In 474
475 the present study, under fucoidan intake, the ingestion of H. pylori 476
477 was reduced and the resistance of the nematode to this 478
479 pathogen was improved (longer lifespan, improved fertility 470
478 rate). Under fucoidan ingestion, the ingestion of C. elegans 479
480 was reduced and the resistance of the nematode to this 481
482 pathogen was improved (longer lifespan, increased fertility 483
484 rate). Under fucoidan ingestion, C. elegans recovered the 485
486 capability to grow even in the presence of H. pylori, up to 487
488 levels corresponding to the pattern of nematodes fed E. coli 489
490 OP50. Similar results were detected for the fertility assays; 491
492 increasing the RS was increased close to 5-fold due to the 493
494 addition of 100 µg/ml fucoidan to the media addition to the 495
496 media. Confirming the lower values of H. pylori present in the 497
498 nematode fed under fucoidan exposure, it was assumed that 499
500 there was a possible combined protective effect between 501
502 the antioxidant and fucoidan by signaling specific defense 503
504 pathways in the nematode (e.g avoiding the ingestion of H. 505
506 pylori) and the effective antibacterial potential of this 507
508 compound exerted on H. pylori cells (anti-adherence and 509
510 bactericidal activity at the in vivo level).53,54 According to Ewald 511
512 (2018)54, reactive oxygen species (ROS) and antioxidant intake 513
514 homeostasis are important for extracellular matrix integrity, 515
516 pathogen defense, oxidative stress resistance, and longevity in 517
518 C. elegans, probably explaining the synergy between fucoidan 519
520 effects (the direct antioxidant potential, pathogen defense and 521
522 antimicrobial specific potential of this molecule were 523
524 (2017)44 showed significant antimicrobial effects of fucoidan 525
from Spatoglossum asperum at concentrations in the range 526
100-150 µg/ml. Furthermore, Lee et al., (2013)55 previously 527
demonstrated the synergic effect of fucoidan in combination 528
with antibodies, reducing oral pathogenic bacteria. However, 529
no previous study detected a reduction in H. pylori 530
proliferation in culture (bacteriostatic or bactericidal effect) 531
during an exposure time of 24-48 h. In contrast, and according 532
to the results obtained in the present study, it was 533
534 demonstrated that the antimicrobial potential of fucoidan 535
against H. pylori was significantly enhanced by both, the 536
fucoidan concentration added to the medium, and the 537
exposure time (0-7 days). According to the results of Mak et 538
al., (2014)50, the addition of 100 µg/ml fucoidan from Undaria 539
pinnatifida to the medium dislodged H. pylori from host cell 540
surface. Combining the results from both studies, it is possible 541
to infer that under the correct dosage, novel drug 542
development can be carried out, including fucoidan as 543
complement to antibiotics in the treatment of H. pylori 544
infection, with the effectiveness of the treatment optimized 545
based on the ingested fucoidan concentration (anti-adherence 546
/ anti-biofilm / and killing effects on H. pylori), and also 547
basa to treatment exposure time.50,51,52,54

430

In the present study, the in vitro and in vivo concentrations 446
461 levels of fucoidan showing bactericidal potential against 462
468 H. pylori were very low for Undaria pinnatifida (5-100 µg/ml) 463
469 Previous studies by Chua et al., (2015)52 revealed that fucoidan 470
471 from Undaria pinnatifida reduced the adherence of H. pylori 472
477 human gastric adenocarcinoma epithelial cells (AGS) when 478
479 added at a concentration of 100 µg/ml. Also, Palanisamy et 480
483
influenced/improved in the nematode, ) preventing \textit{H. pylori} infection.

\section*{Conclusions}

Fucoidan from \textit{Undaria pinnatifida} had the highest bacteriostatic and bactericidal potential against \textit{H. pylori} at low concentrations, in the range of [5-10] µg/ml. Exposure time was a determining factor reducing \textit{H. pylori} to close to 1 log\textsubscript{10} cycles just after 7 days of exposure at 25 °C. The in vitro antimicrobial potential of fucoidan from \textit{Undaria pinnatifida} was confirmed in \textit{C. elegans}, an \textit{in vivo} model for infection.

\textit{C. elegans} fed \textit{H. pylori}, supplementation of the media with fucoidan (100 µg/ml) increased the \textit{C. elegans} lifespan from 5.57 to 5.97 (NGM) to 26 days (NGM+fucoidan). These results open a promising line of research regarding the development of nutraceutical ingredients derived from fucoidan to be used as complementary therapies to be applied in \textit{H. pylori} infected patients. The beneficial effects associated to this algal ingredient are being extensively reported [2010-2020] and important to highlight the future application (2020-2030) of preservation, pharmaceutical, biotechnological, and medicinal properties of this compound as a sustainable and effective alternative to antimicrobial answering the demand of the WHO regarding the urgent need for agents against concerning antibiotic resistant pathogens.

\section*{Conflicts of interest}

In accordance with our policy on \textit{Conflicts of interest} please ensure that a conflicts of interest statement is included in your manuscript here. Please note that this statement is required for all submitted manuscripts. If no conflicts exist, please state that “There are no conflicts to declare”.

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