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Fucoidan but not 2'-Fucosyllactose could inhibit human norovirus replication in zebrafish larvae

Malcolm Turk Hsern Tan¹, Yan Li², Mohamad Eshaghi Gorji¹, Zhiyuan Gong², Dan Li^{1*}

¹ Department of Food Science & Technology, Faculty of Science, National University of Singapore, Singapore, Singapore
² Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore

* Corresponding author: Dr. Dan Li (ORCID: 0000-0001-6645-2489)
fstlda@nus.edu.sg

22 **Abstract**

23 Human noroviruses (hNoVs) cause heavy disease burden worldwide and there is no clinically
 24 approved vaccination or antivirals hitherto. In this study, with the use of a zebrafish larvae *in*
 25 *vivo* platform, we investigated the anti-hNoV potentials of fucoidan and 2'-Fucosyllactose
 26 (2'-FL). As a result, although both fucoidan and 2'-FL were able to block hNoV GII.4 virus-like
 27 particle (VLPs) from binding to type A saliva as expected, only fucoidan but not 2'-FL was
 28 able to inhibit the replication of hNoV GII.P16-GII.4 in zebrafish larvae, indicating the
 29 possible needs of higher molecular weights for fucosylated carbohydrates to exert anti-
 30 hNoV effect.

31

32 **Key words**

33 Norovirus, fucoidan, 2'-Fucosyllactose, antiviral

34 **1. Introduction**

35 Human noroviruses (hNoVs) are increasingly identified as an important cause of acute
 36 gastroenteritis outbreaks associated with great disease burden worldwide. In 2015, the
 37 World Health Organization (WHO) estimated that 684 million diarrheal disease cases were
 38 caused by hNoVs annually, amongst which 212,000 deaths occurred (WHO, 2015). Since
 39 hNoVs are frequently linked with foodborne outbreaks, it is also regarded as the “Number 1”
 40 cause of foodborne illnesses (Iturriza-Gomara & O’Brien, 2016; Marsh et al., 2018; WHO,
 41 2015). Similar to many other viruses, hNoV caused infection is usually self-limiting within a
 42 few days for most healthy people, while severe and long-lasting symptoms and even death
 43 could be induced in vulnerable population (Chong & Atmar, 2019).

44 Vaccination, despite of significant research efforts dedicated for many years, remains
 45 infeasible as hNoVs evolve extremely rapidly (Hallowell et al., 2019). As for antivirals, none
 46 has yet been clinically approved, and the majority of candidates are still in the early stages
 47 of preclinical development as reviewed by Netzler et al. (2018). Thus, there is urgent need
 48 to screen for alternative solutions based on solid scientific evidence to mitigate NoV
 49 infection especially for the vulnerable population.

50 Since histo-blood group antigens (HBGA) have been shown to have close correlations with
 51 hNoV infection (Tan & Jiang, 2005; Singh et al., 2016), the *in vitro* HBGA-binding block assay
 52 has been used in many studies to screen for anti-hNoV candidates (Ali et al., 2016; Derya et
 53 al., 2020; Zhang et al., 2012; 2013). As hNoV cultivation remains to be challenging (Ettayebi
 54 et al., 2016), not many of the candidates have been further validated. In 2019, a zebrafish
 55 (*Danio rerio*) larva platform has been reported to be able to support replication of multiple
 56 hNoV strains (Van Dycke et al., 2019). This is of great interest for its feasibility of high-

throughput and cost-effectiveness. In fact, zebrafish, which share remarkable genetic, physiologic and pharmacologic similarities to humans (Goldsmith & Jobin, 2012), have been used as a versatile research organism that can be experimentally infected with many viruses (Burgos et al., 2008; Ding et al., 2011; Gabor et al., 2014). It has been proposed to be used for the discovery of new viruses recently (Balla et al., 2020).

In this study, we applied the zebrafish larvae platform to evaluate the anti-hNoV effect of fucoidan and 2'-Fucosyllactose (2'-FL). Both carbohydrates have been reported as putative anti-hNoV candidates due to their fucose contents and thus specific hNoV binding ability (Hanisch et al., 2018; Kim et al., 2020; Koromyslova et al., 2017; Schrotten et al., 2016; Weichert et al., 2016). Specifically, 2'-FL was observed to bind to NoV GII.4 and GII.10 capsid at the same pockets with HBGA (Schrotten et al., 2016). Fucoidan was proposed as a more efficient anti-NoV candidate due to its fucose multivalency (Hanisch et al., 2018).

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2. Materials and methods

2.1 Ethics statement

All zebrafish experiments were performed in compliance with the Institutional Animal Care and Use Committee guidelines, National University of Singapore.

HNoVs in stool samples were kindly provided by Singapore General Hospital.

Type A saliva samples were collected from healthy adults. The human milk sample was donated by a blood type A woman during her breast-feeding period. The collection of both human saliva and human milk samples were under the ethical approval of National University of Singapore Institutional Review Board (NUS-IRB, reference code: N-20-035).

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80 2.2 HNoV virus-like particle (VLP) binding block assay

81 Type A saliva samples were boiled at 95°C for 10 min, followed by centrifugation (Eppendorf,
82 Hamburg, Germany) at 10,000 × g for 5 min. The supernatants were diluted at 1:1500 by
83 carbonate : bicarbonate buffer (pH 9.4) and used to coat 96-well microtiter plates (100
84 µl/well) at 4°C overnight. After being washed once with phosphate-buffered saline (PBS)
85 containing 0.05% Tween 20 (PBS-T), the plate was blocked with 5% nonfat dried milk (Blotto,
86 200 µl/well) at 37°C for 1 h. NoV GII.4 VLPs (1.1 mg/ml, purity greater than 95%, The Native
87 Antigen Company, UK) were pre-incubated with the fucoidan (from *Fucus vesiculosus*,
88 Sigma-Aldrich), 2'-FL (Sigma-Aldrich) or human milk at room temperature for 1 h, diluted
89 with 5% nonfat dried milk to a final VLP concentration of 1 µg/ml and added to the plates at
90 100 µl/well for incubation at 37°C for 1 h. After being washed three times with PBS-T, the
91 bound NoV capsid proteins were detected with mouse anti norovirus GII (1:1000;
92 NativeAntigen) and by adding horseradish peroxidase conjugated with goat anti-mouse IgG
93 (1:1500; Sigma Aldrich). Horseradish peroxidase activity was detected with a TMB (3,3',5,5'-
94 tetramethylbenzidine) kit (Sigma Aldrich), and the signal intensities (the optical density at
95 450 nm [OD₄₅₀]) were read with a Multiskan Sky plate reader (Thermo Fisher Scientific).
96 OD₄₅₀ of the blank controls were below 0.10.

98 2.3 Zebrafish maintenance and injection of zebrafish larvae with hNoV in the yolk

99 Wild type adult zebrafish were maintained in the aquatic facility of National University of
100 Singapore with water temperature of 28°C and 14/10 h light/dark cycle. Fertilized eggs were
101 collected from adults placed in mating cages and kept in petri dishes containing embryo
102 medium E3 (5mM of NaCl, 0.17 mM KCl, 0.33 mM of CaCl₂, 0.33 mM MgSO₄).

The two NoV GII samples used in this study were genotyped as GII.P16-GII.4 and GII.P31 (GII.Pe)-GII.17 over the B-C genome region as reported by Anderson et al. (2001) and Kojima et al. (2002) and with the use of Norovirus Automated Genotyping Tool developed by Kroneman et al. (2011). In order to prepare the virus suspension for injecting to zebrafish larvae, an aliquot of 100 mg of each stool sample was suspended in 1 ml of sterile PBS, thoroughly vortex and centrifuged for 5 min at 9, 000 × g; the supernatant was harvested and stored at -80°C until further use.

The injection was performed according to Van Dycke et al., (2019) with slight modifications. Briefly, 3 days post-fertilization (dpf) zebrafish larvae were anaesthetized for 2 - 3 minutes in embryo medium E3 containing 0.2 mg/ml Tricaine (Sigma-Aldrich). Thereafter, the anaesthetized zebrafish larvae were transferred to a petri dish with grooves of a mold imprint (6 rows of V-shaped grooves) in 1.5% agarose, aligned so that they were lying on their dorsal side with the yolk facing upwards. Three nL of the viral suspension (with or without pre-incubation with fucoidan or 2'-FL at room temperature for 1 h) was injected into the yolk of each zebrafish larva with a pulled borosilicate glass capillary needle. After the injection, the zebrafish larvae were transferred to a petri dish containing E3 and further maintained at 29.5°C. At 1, 48 and 72 hours post-injection (hpi), regarded as 0, 2 and 3 days post-injection (dpi), the zebrafish larvae were harvested with 10 larvae pooled as one sample, washed and collected in 2 ml tubes containing 0.5 mm yttria-stabilized zirconium oxide beads, Lysing Matrix Y (MP Biomedicals, USA).

2.4 Tissue homogenization, RNA extraction and RT-qPCR for detection of hNoV

The zebrafish larvae collected in Lysing Matrix Y tubes were homogenized with 3 cycles of 15 s at 6,500 RPM with rest intervals of 60 s by FastPrep™ 24-5G tissue-cell homogenizer (MP Biomedicals, USA). The homogenates were clarified by centrifugation at 9,000 × g for 5 min and RNA was extracted using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol.

RT-qPCR analyses of NoV GII were carried out using GoTaq® Probe 1-Step RT-qPCR System (Promega, USA). Primers and the FAM/TAMRA-labelled probe of NoV GII were according to ISO 15216-1:2017. Forward primer QNIF2: 5'-ATGTTTCAGRTGGATGAGRTTCTCWGA-3'; reverse primer G2SKR: 5'-TCGACGCCATCTTCATTACACA-3'; probe QNIFs: 5' FAM-AGCACGTGGGAGGGCGATCG-3' TAMRA. Cycling conditions were 45°C for 15 min, then 95°C for 10 min, followed by 40 cycles with 95°C for 15 s and 60°C for 30 s in each cycle. Cycle threshold (Ct) values were determined during RT-qPCR analysis using StepOne™ system (Applied Biosystems, USA).

Double-stranded DNA (dsDNA) containing the specific primers-probe binding sites were synthesized for NoV GII and cloned into the pGEM-T Vector (Promega) resulting in the NoV-GII plasmids. The plasmids with inserts were purified by using a Plasmid Midi Kit (Qiagen). The plasmid concentration was determined by photospectroscopy at 260 nm using the BioDrop Duo™ spectrophotometer (BioDrop, United Kingdom). Ten-fold serial dilutions ranging from 5 × 10⁶ to 5 copies of all positive control plasmids were used to prepare a standard curve and enumerate the NoV GII detected from the zebrafish larvae.

2.5 Statistical analysis

Statistical analyses were performed using the SPSS 22 software for Windows (SPSS, Inc., Chicago, IL, USA). Student's t-tests were performed on the VLPs binding block assay, and the

nonparametric Mann-Whitney U tests were performed on the *in vivo* zebrafish model.

Significant differences were considered when P was <0.05.

3. Results

3.1 Both fucoidan and 2'-FL were able to block the binding of hNoV VLPs to HBGAs in type

A saliva

Pre-incubation of hNoV GII.4 VLPs with both fucoidan and 2'-FL were able to decrease the binding affinity of hNoV GII.4 VLPs to type A saliva coated on plates in a dose-dependent manner (Figure 1). At both concentrations of 2.40 mg/ml and 24.00 mg/ml, the reducing effects induced by 2'-FL were significantly ($P < 0.05$) more prominent than fucoidan. Last, a human milk sample collected from a type A secretor showed more efficient binding block effect than 24.00 mg/ml of fucoidan and 2'-FL (Figure 1).

3.2 HNoV GII.P16-GII.4 replicated in zebrafish larvae more consistently than GII.P31-GII.17 at 2 dpi

In order to validate if the zebrafish larvae could indeed support the replication of hNoVs with our experimental set-up and hNoV samples, two NoV genogroup II clinical (stool) samples belonging to GII.P16-GII.4 (8-log genomic copies/g stool) and GII.P31-GII.17 (9-log genomic copies/g stool) were tested by injecting 3 nl of the virus suspension in the yolk of each larva at 3 dpf. At 0, 2 and 3 dpi, 10 injected larvae were pooled as one sample and proceeded to homogenization, RNA extraction and RT-qPCR for hNoV GII detection.

As a result, for hNoV GII.P16-GII.4, a low injection dose of ~2-log genomic copies per larva (detected as <2-log genomic copies per sample as shown in Table 1A) could lead to consistent virus replication to >4-log genomic copies per sample at 2 dpi for all tested five samples in four independent experiments (Table 1A). At 3 dpi, two out of the five samples dropped with >2-log genomic copies/sample and thus in the following studies, 2 dpi was selected as the time point to evaluate the anti-hNoV effect for hNoV GII.P16-GII.4.

As for GII.P31-GII.17, although it was able to start with higher injection doses due to the higher virus loads in the stool sample, replication of only ~1-log genomic copies/sample was observed in two out of the five samples while no viral replication was observed for the rest (Table 1B), showing that GII.P31-GII.17 is less prominent than GII.P16-GII.4 to be used to evaluate the anti-hNoV effect.

3.3 Fucoidan but not 2'-FL inhibited hNoV GII.P16-GII.4 replication in zebrafish larvae

The effects of fucoidan and 2'-FL on hNoV replication in zebrafish larvae were investigated starting with 24.00 mg/ml, as pre-incubation with both carbohydrates at this concentration induced significant reductions ($P < 0.05$) of NoV GII.4 VLPs binding to type A saliva (Figure 1). However, 24.00 mg/ml and 12.00 mg/ml fucoidan showed toxicity when injected to the zebrafish larvae and the larvae were only able to maintain the survival rates above 90% at 2 dpi when injected with low concentrations of fucoidan from 6.00 mg/ml downwards.

At 2 dpi, the zebrafish larvae groups injected with hNoV GII.P16-GII.4 suspension pre-incubated with 6.00 mg/ml and 2.40 mg/ml fucoidan showed no virus replication as the RT-qPCR detection results for hNoV GII were below the detection limit (<1.3-log genomic

copies/sample), whereas lower concentration of fucoidan at 0.24 mg/ml as well as the positive control group still supported viral replication to >4-log genomic copies/sample (Table 2A).

As for 2'-FL, when pre-incubated with hNoV GII.P16-GII.4 suspension and injected to the zebrafish larvae, no inhibition effect was observed even at the high concentration of 24.00 mg/ml (Table 2B).

Next, we selected the highest possible concentration at 6.00 mg/ml to test the effect fucoidan and 2'-FL in parallel. During all four independent experiments performed in four different weeks, the groups of 6.00 mg/ml fucoidan were three times detected with below detection limit (<1.3-log genomic copies/sample) and once slightly above (1.8-log genomic copies/sample), and the groups of 6.00 mg/ml 2'-FL did not show any significant inhibition in comparison with the positive controls ($P>0.05$) (Figure 2).

3.4 Saliva but not human milk from type A secretors inhibited hNoV GII.P16-GII.4 replication in zebrafish larvae

The saliva and human milk used in this study were collected from both type A secretors. The human milk was able to block the binding of NoV GII.4 VLPs to saliva even more efficiently than 24.00 mg/ml fucoidan and 2'-FL (Figure 1). When the hNoV GII.P16-GII.4 viral suspensions were pre-incubated with the saliva before injecting to the zebrafish larvae, no virus replication was observed (2.4-log genomic copies/sample at 0 dpi and 2.2-log genomic copies/sample at 2 dpi), whereas the group pre-incubated with human milk as well as the positive control groups still supported viral replication to >4-log genomic copies/sample.

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215 4. Discussion

216 Abundant evidence from dedicated research has shown that recognition of HBGAs is
 217 essential for hNoV to cause infection (Nordgren & Svensson, 2019; Singh et al., 2016; Tan &
 218 Jiang, 2005). As α -fucose has been determined to be an important glycan epitope of HBGAs
 219 binding to hNoVs (Hanisch et al., 2018), fucosylated carbohydrates including both fucoidan
 220 and 2'-FL have been reported to be able to inhibit the binding of hNoVs to HBGAs and thus
 221 proposed as potential anti-hNoV candidates (Derya et al., 2020). Fucoidan is a fucose-
 222 containing sulphated polysaccharide commonly found from brown algae with widely
 223 reported bioactivities such as antioxidant, anticancer, antiviral and antibacterial activities
 224 (Luthuli et al., 2019). Recently, Kim et al. (2020) have found that oral administration of
 225 *Laminaria japonica* fucoidan to STAT1^{-/-} mice was able to improve their survival rates from
 226 murine norovirus infection and reduce virus loads in their feces. 2'-FL is one of the most
 227 recognized human milk oligosaccharides (Goehring et al., 2016) and has been applied in
 228 infant formula products on the markets (Schott, 2016). The structural basis between 2'-FL
 229 and hNoVs have been clearly elucidated (Koromyslova et al., 2017; Schroten et al., 2016;
 230 Weichert et al., 2016), which is believed to be the theoretical foundation to explain the
 231 protective effects of breast milk against hNoV infection. Therefore, both fucoidan and 2'-FL
 232 were tested in this study with the novel in vivo zebrafish larva platform in order to further
 233 investigate their anti-hNoV activities.

234 According to our results, although both fucoidan and 2'-FL were able to block hNoV GII.4
 235 VLPs binding to type A saliva as expected, only fucoidan but not 2'-FL was able to inhibit the
 236 replication of hNoV GII.P16-GII.4 in zebrafish larvae. To the best of our knowledge, this is

the first time showing that blocking of HBGA binding sites *in vitro* may not necessarily inhibit hNoV replication *in vivo*. As an explanation, we assume that fucoidan, with much higher molecular weight than 2'-FL, may supply a much more complete "shield" for the virions to prevent the interactions between the virus with other downstream receptors/co-factors; whereas 2'-FL binding to the HBGA binding pockets did not manifest such effect. Although HBGA is shown to have a close correlation with hNoV infection, the pathogenesis of hNoVs is still under investigation (Green et al., 2020) and the exact role that HBGAs play in the hNoV infection is still largely unknown. Several proteins from human biological components have been reported with hNoV affinity, suggesting that there could be other essential receptors next to HBGA involved in hNoV infection (Almand et al., 2017). Our result supports this point of view from a brand new angle. One may argue that the infection mechanism in zebrafish larvae may differ from infection in human, which is indeed pending to be studied in-depth in the future. However, as the virus used in this study is a genuine hNoV strain with clinical evidence from the supplier, there is a high chance that the zebrafish larvae share similarities in the routes supporting hNoV replication.

In order to further test the correlation between NoV-HBGA binding and NoV replication inhibition, we tested saliva and human milk, the two biological samples with known affinities to hNoVs. When pre-incubated with hNoV GII.P16-GII.4 and injected to zebrafish larvae, only the saliva but not the human milk sample tested in this study (collected from type A secretors) was able to inhibit the replication of hNoV. Saliva and human milk are complicated mixtures with not only oligosaccharides but more importantly glycoproteins (Ruvoën-Clouet et al., 2006). Although both contain HBGAs, the carbohydrate profiles of saliva and human milk have been indeed reported to be different (Jiang et al., 2004). In this study, it seems only the saliva but not the human milk sample was able to shield the hNoV

virions from being recognized by the downstream receptor/co-factors causing infection in the zebrafish larvae, which remain to be revealed in the future. It must be noticed that this result may still vary depending on the phenotype of the biological sample donors. The data presented in this study is only to complement the results of fucoidan and 2'-FL, demonstrating the discrepancies between HBGA-NoV affinity and NoV replicating inhibition in zebrafish larvae.

In summary, with the use of a zebrafish larva platform, this study validated the anti-hNoV activity of fucoidan originated from brown algae *Fucus vesiculosus* and demonstrated the possible needs of higher molecular weights other than oligosaccharides such as 2'-FL for fucosylated carbohydrates to exert anti-hNoV effect. The zebrafish larva tool has shown excellent sensitivity in our hands as we have been using a virus stool sample (GII.P16-GII.4) with much lower virus load ($\sim 10^8$ genomic copies/g stool) in comparison with the report by Van Dycke et al. (2019), of which the stool samples were with virus loads generally higher than 10^{10} genomic copies/g stool. This zebrafish larva platform still suffers from a few limitations such as short-duration infection which only lasts for a few days and infeasibility of virus inoculation via immersion (Van Dycke et al., 2019). Thus, it is not possible to test more time points of fucoidan administration to mimic better the genuine scenarios of human infection and protection. However, with the use of an *in vivo* model, especially by showing the controversial results between *in vivo* and *in vitro* tests of 2'-FL, this study serves as one step forward in exploring potential anti-hNoV candidates with more solid evidence.

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References

Ali, E. S., Rajapaksha, H., Carr, J. M., & Petrovsky, N. (2016). Norovirus drug candidates that inhibit viral capsid attachment to human histo-blood group antigens. *Antiviral Research*, 133, 14–22. <https://doi.org/10.1016/j.antiviral.2016.07.006>

Almand, E. A., Moore, M. D., & Jaykus, L.-A. (2017). Norovirus Binding to Ligands Beyond Histo-Blood Group Antigens. *Frontiers in Microbiology*, 8, 2549. <https://doi.org/10.3389/fmicb.2017.02549>

Anderson, A. D. (2001). Multistate Outbreak of Norwalk-like Virus Gastroenteritis Associated with a Common Caterer. *American Journal of Epidemiology*, 154(11), 1013–1019. <https://doi.org/10.1093/aje/154.11.1013>

Balla, K. M., Rice, M. C., Gagnon, J. A., & Elde, N. C. (2020). Linking Virus Discovery to Immune Responses Visualized during Zebrafish Infections. *Current Biology*, 30(11), 2092–2103.e5. <https://doi.org/10.1016/j.cub.2020.04.031>

Burgos, J. S., Ripoll-Gomez, J., Alfaro, J. M., Sastre, I., & Valdivieso, F. (2008). Zebrafish as a New Model for Herpes Simplex Virus Type 1 Infection. *Zebrafish*, 5(4), 323–333. <https://doi.org/10.1089/zeb.2008.0552>

- 303 Chong, P. P., & Atmar, R. L. (2019). Norovirus in health care and implications for the
304 immunocompromised host. *Current Opinion in Infectious Diseases*, 32(4), 348–355.
305 <https://doi.org/10.1097/qco.0000000000000557>
- 306 Derya, S. M., Spiegel, H., Hanisch, F.-G., Morozov, V., Schrotten, H., Jennewein, S., &
307 Parschat, K. (2020). Biotechnologically produced fucosylated oligosaccharides inhibit
308 the binding of human noroviruses to their natural receptors. *Journal of*
309 *Biotechnology*, 318, 31–38. <https://doi.org/10.1016/j.jbiotec.2020.05.001>
- 310 Ding, C.-B., Zhang, J.-P., Zhao, Y., Peng, Z.-G., Song, D.-Q., & Jiang, J.-D. (2011). Zebrafish as a
311 Potential Model Organism for Drug Test Against Hepatitis C Virus. *PLoS ONE*, 6(8),
312 e22921. <https://doi.org/10.1371/journal.pone.0022921>
- 313 Ettayebi, K., Crawford, S. E., Murakami, K., Broughman, J. R., Karandikar, U., Tenge, V. R.,
314 Neill, F. H., Blutt, S. E., Zeng, X.-L., Qu, L., Kou, B., Opekun, A. R., Burrin, D., Graham,
315 D. Y., Ramani, S., Atmar, R. L., & Estes, M. K. (2016). Replication of human
316 noroviruses in stem cell-derived human enteroids. *Science*, 353(6306), 1387–1393.
317 <https://doi.org/10.1126/science.aaf5211>
- 318 Gabor, K. A., Goody, M. F., Mowel, W. K., Breitbart, M. E., Gratacap, R. L., Witten, P. E., &
319 Kim, C. H. (2014). Influenza A virus infection in zebrafish recapitulates mammalian
320 infection and sensitivity to anti-influenza drug treatment. *Disease Models &*
321 *Mechanisms*, 7(11), 1227–1237. <https://doi.org/10.1242/dmm.014746>
- 322 Green, K. Y., Kaufman, S. S., Nagata, B. M., Chaimongkol, N., Kim, D. Y., Levenson, E. A., Tin,
323 C. M., Yardley, A. B., Johnson, J. A., Barletta, A. B. F., Khan, K. M., Yazigi, N. A.,

- 324 Subramanian, S., Moturi, S. R., Fishbein, T. M., Moore, I. N., & Sosnovtsev, S. V.
 325 (2020). Human norovirus targets enteroendocrine epithelial cells in the small
 326 intestine. *Nature Communications*, 11(1), 2759. [https://doi.org/10.1038/s41467-](https://doi.org/10.1038/s41467-020-16491-3)
 327 020-16491-3
- 328 Goehring, K. C., Marriage, B. J., Oliver, J. S., Wilder, J. A., Barrett, E. G., & Buck, R. H. (2016).
 329 Similar to Those Who Are Breastfed, Infants Fed a Formula Containing 2'-
 330 Fucosyllactose Have Lower Inflammatory Cytokines in a Randomized Controlled Trial.
 331 *The Journal of Nutrition*, 146(12), 2559–2566.
 332 <https://doi.org/10.3945/jn.116.236919>
- 333 Goldsmith, J. R., & Jobin, C. (2012). Think Small: Zebrafish as a Model System of Human
 334 Pathology. *Journal of Biomedicine and Biotechnology*, 2012, 1–12.
 335 <https://doi.org/10.1155/2012/817341>
- 336 Hallowell, B. D., Parashar, U. D., & Hall, A. J. (2018). Epidemiologic challenges in norovirus
 337 vaccine development. *Human Vaccines & Immunotherapeutics*, 15(6), 1279–1283.
 338 <https://doi.org/10.1080/21645515.2018.1553594>
- 339 Hanisch, F.-G., Hansman, G. S., Morozov, V., Kunz, C., & Schroten, H. (2018). Avidity of α -
 340 fucose on human milk oligosaccharides and blood group-unrelated
 341 oligo/polyfucoses is essential for potent norovirus-binding targets. *Journal of*
 342 *Biological Chemistry*, 293(30), 11955–11965.
 343 <https://doi.org/10.1074/jbc.ra117.001369>

344 International Organization for Standardization (2017). ISO 15216-1 Microbiology of food and
345 animal feed. Horizontal method for determination of Hepatitis A virus and norovirus
346 in food using real-time RT-PCR. Part 1: Method for quantification.

347 Iturriza-Gomara, M., & O'Brien, S. J. (2016). Foodborne viral infections. *Current Opinion in*
348 *Infectious Diseases*, 29(5), 495–501.
349 <https://doi.org/10.1097/qco.0000000000000299>

350 Jiang, X., Huang, P., Zhong, W., Tan, M., Farkas, T., Morrow, A.L., Newburg, D.S., Ruiz-
351 Palacios, G.M., & Pickering, L.K. (2004). Human milk contains elements that block
352 binding of noroviruses to human histo-blood group antigens in saliva. *The Journal of*
353 *Infectious Diseases*, 190(10), pp.1850-1859.

354 Kim, H., Lim, C. Y., Lee, D. B., Seok, J. H., Kim, K. H., & Chung, M. S. (2020). Inhibitory Effects
355 of *Laminaria japonica* Fucoidans Against Noroviruses. *Viruses*, 12(9), 997.
356 <https://doi.org/10.3390/v12090997>

357 Kojima, S., Kageyama, T., Fukushi, S., Hoshino, F. B., Shinohara, M., Uchida, K., Natori, K.,
358 Takeda, N., & Katayama, K. (2002). Genogroup-specific PCR primers for detection of
359 Norwalk-like viruses. *Journal of Virological Methods*, 100(1–2), 107–114.
360 [https://doi.org/10.1016/s0166-0934\(01\)00404-9](https://doi.org/10.1016/s0166-0934(01)00404-9)

361 Koromyslova, A., Tripathi, S., Morozov, V., Schroten, H., & Hansman, G. S. (2017). Human
362 norovirus inhibition by a human milk oligosaccharide. *Virology*, 508, 81–89.
363 <https://doi.org/10.1016/j.virol.2017.04.032>

364 Kroneman, A., Vennema, H., Deforche, K., Avoort, H., Peñaranda, S., Oberste, M. S., Vinjé, J.,
365 & Koopmans, M. (2011). An automated genotyping tool for enteroviruses and
366 noroviruses. *Journal of Clinical Virology*, 51(2), 121–125.
367 <https://doi.org/10.1016/j.jcv.2011.03.006>

368 Luthuli, S., Wu, S., Cheng, Y., Zheng, X., Wu, M., & Tong, H. (2019). Therapeutic Effects of
369 Fucoidan: A Review on Recent Studies. *Marine Drugs*, 17(9), 487.
370 <https://doi.org/10.3390/md17090487>

371 Marsh, Z., Shah, M. P., Wikswo, M. E., Barclay, L., Kisselburgh, H., Kambhampati, A., Cannon,
372 J. L., Parashar, U. D., Vinjé, J., & Hall, A. J. (2018). Epidemiology of Foodborne
373 Norovirus Outbreaks – United States, 2009–2015. *Food Safety*, 6(2), 58–66.
374 <https://doi.org/10.14252/foodsafetyfscj.2017028>

375 Netzler, N. E., Enosi Tuipulotu, D., & White, P. A. (2018). Norovirus antivirals: Where are we
376 now? *Medicinal Research Reviews*, 39(3), 860–886.
377 <https://doi.org/10.1002/med.21545>

378 Nordgren, J., & Svensson, L. (2019). Genetic Susceptibility to Human Norovirus Infection: An
379 Update. *Viruses*, 11(3), 226. <https://doi.org/10.3390/v11030226>

380 Ruvoën-Clouet, N., Mas, E., Marionneau, S., Guillon, P., Lombardo, D., & Le Pendu, J. (2006).
381 Bile-salt-stimulated lipase and mucins from milk of ‘secretor’ mothers inhibit the
382 binding of Norwalk virus capsids to their carbohydrate ligands. *Biochemical*
383 *Journal*, 393(3), pp.627-634.

- 384 Schott M (2016). *New Study: Babies Fed Infant Formula with 2'-FL Human Milk*
 385 *Oligosaccharide+ Had Immune Response More Like Breastfed Babies*. Abbott
 386 MediaRoom. [https://abbott.mediaroom.com/2016-10-26-New-Study-Babies-Fed-](https://abbott.mediaroom.com/2016-10-26-New-Study-Babies-Fed-Infant-Formula-with-2-FL-Human-Milk-Oligosaccharide-Had-Immune-Response-More-Like-Breastfed-Babies)
 387 [Infant-Formula-with-2-FL-Human-Milk-Oligosaccharide-Had-Immune-Response-](https://abbott.mediaroom.com/2016-10-26-New-Study-Babies-Fed-Infant-Formula-with-2-FL-Human-Milk-Oligosaccharide-Had-Immune-Response-More-Like-Breastfed-Babies)
 388 [More-Like-Breastfed-Babies](https://abbott.mediaroom.com/2016-10-26-New-Study-Babies-Fed-Infant-Formula-with-2-FL-Human-Milk-Oligosaccharide-Had-Immune-Response-More-Like-Breastfed-Babies)
- 389 Schroten, H., Hanisch, F.-G., & Hansman, G. S. (2016). Human Norovirus Interactions with
 390 Histo-Blood Group Antigens and Human Milk Oligosaccharides. *Journal of Virology*,
 391 *90*(13), 5855–5859. <https://doi.org/10.1128/jvi.00317-16>
- 392 Singh, B. K., Leuthold, M. M., & Hansman, G. S. (2016). Structural Constraints on Human
 393 Norovirus Binding to Histo-Blood Group Antigens. *MSphere*, *1*(2), e00049 – 16.
 394 <https://doi.org/10.1128/msphere.00049-16>
- 395 Tan, M., & Jiang, X. (2005). Norovirus and its histo-blood group antigen receptors: an answer
 396 to a historical puzzle. *Trends in Microbiology*, *13*(6), 285–293.
 397 <https://doi.org/10.1016/j.tim.2005.04.004>
- 398 Van Dycke, J., Ny, A., Conceição-Neto, N., Maes, J., Hosmillo, M., Cuvry, A., Goodfellow, I.,
 399 Nogueira, T. C., Verbeken, E., Matthijnsens, J., de Witte, P., Neyts, J., & Rocha-
 400 Pereira, J. (2019). A robust human norovirus replication model in zebrafish larvae.
 401 *PLOS Pathogens*, *15*(9), e1008009. <https://doi.org/10.1371/journal.ppat.1008009>
- 402 Weichert, S., Koromyslova, A., Singh, B. K., Hansman, S., Jennewein, S., Schroten, H., &
 403 Hansman, G. S. (2016). Structural Basis for Norovirus Inhibition by Human Milk

404 Oligosaccharides. *Journal of Virology*, 90(9), 4843–4848.
405 <https://doi.org/10.1128/jvi.03223-15>

406 WHO (World Health Organization). (2015). WHO Estimates of the Global Burden of
407 Foodborne Diseases: Foodborne Disease Burden Epidemiology Reference Group
408 2007–2015.

409 Zhang, X.-F., Dai, Y.-C., Zhong, W., Tan, M., Lv, Z.-P., Zhou, Y.-C., & Jiang, X. (2012). Tannic
410 acid inhibited norovirus binding to HBGA receptors, a study of 50 Chinese medicinal
411 herbs. *Bioorganic & Medicinal Chemistry*, 20(4), 1616–1623.
412 <https://doi.org/10.1016/j.bmc.2011.11.040>

413 Zhang, X.-F., Tan, M., Chhabra, M., Dai, Y.-C., Meller, J., & Jiang, X. (2013). Inhibition of
414 Histo-blood Group Antigen Binding as a Novel Strategy to Block Norovirus Infections.
415 *PLoS ONE*, 8(7), e69379. <https://doi.org/10.1371/journal.pone.0069379>

Figure 1 Percentage (%) of NoV GII.4 VLPs pre-incubated with fucoidan, 2'-FL or human milk binding to type A saliva (OD₄₅₀) in comparison to positive control (NoV GII.4 VLPs without carbohydrate blocking). Each column represents the average of triplicates, and each error bar indicates the standard deviations.

422

423 **Figure 2** Detection of hNoV GII.P16-GII.4 with and without pre-incubation with fucoidan or
 424 2'-FL from zebrafish larva at 0 and 2 dpi. Each sample contains a pool of 10 larvae. Each
 425 column represents the average of four independent experiments conducted in four
 426 different weeks, and each error bar indicates the standard deviations. *, below the limit of
 427 detection (1.3 log genomic copies/sample). **, two out of three samples were below the
 428 limit of detection.

429

Table 1 Detection of hNoV GII.P16-GII.4 **(A)** and GII.P31-GII.17 **(B)** in zebrafish larvae (log genomic copies/sample) at different days post injection (dpi). Each sample contains a pool of 10 larvae. The limit of detection was 1.3 log genomic copies/sample.

(A)

Batch	Sample	Log genomic copies/sample		
		0 dpi	2 dpi	3 dpi
Batch 1	Sample 1	1.7	4.7	4.7
	Sample 2	1.5	4.4	4.0
Batch 2	Sample 1	1.9	4.6	4.8
Batch 3	Sample 1	<1.3	4.8	2.5
Batch 4	Sample 1	<1.3	4.9	2.6

(B)

Batch	Sample	Log genomic copies/sample		
		0 dpi	2 dpi	3 dpi
Batch 1	Sample 1	2.5	n.a.	3.4
	Sample 2	2.5	n.a.	2.3
Batch 2	Sample 1	4.3	n.a.	4.4
Batch 3	Sample 1	2.8	2.9	n.a.
Batch 4	Sample 1	3.0	4.8	n.a.

Table 2 Detection of hNoV GII.P16-GII.4 pre-incubated with different concentrations of fucoidan (A), 2'-FL (B), saliva (C) and human milk (D) in zebrafish larvae (log genomic copies/sample). Each sample represents a pool of 10 larvae. The limit of detection was 1.3 log genomic copies/sample.

(A)

Concentration of fucoidan	Zebrafish larvae survival rate at 2 dpi	Log genomic copies/sample	
		0 dpi	2 dpi
24.0 mg/ml	<90%	<1.3	n.a.
12.0 mg/ml	<90%	<1.3	n.a.
6.00 mg/ml	>90%	<1.3	<1.3
2.40 mg/ml	>90%	<1.3	< 1.3
0.24 mg/ml	>90%	<1.3	4.3
0	>90%	<1.3	4.7

(B)

Concentration of 2'-FL	Zebrafish larvae survival rate at 2 dpi	Log genomic copies/sample	
		0 dpi	2 dpi
24.0 mg/ml	>90%	1.4	5.3
6.00 mg/ml	>90%	1.9	3.9
2.40 mg/ml	>90%	2.3	4.2
0	>90%	1.9	4.1

(C)

	Zebrafish larvae survival rate at 2 dpi	Log genomic copies/sample	
		0 dpi	2 dpi
Saliva (type A secretor)	>90%	2.4	2.2
Control	>90%	1.9	4.1

(D)

	Zebrafish larvae survival rate at 2 dpi	Log genomic copies/sample	
		0 dpi	2 dpi
Human milk (type A secretor)	>90%	<1.3	5.7
Control	>90%	<1.3	5.4