

## Supplementary Information

### **Synthesis of fucosyllactose using $\alpha$ -L-fucosidases GH29 from infant gut microbial metagenome**

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Applied Microbiology and Biotechnology

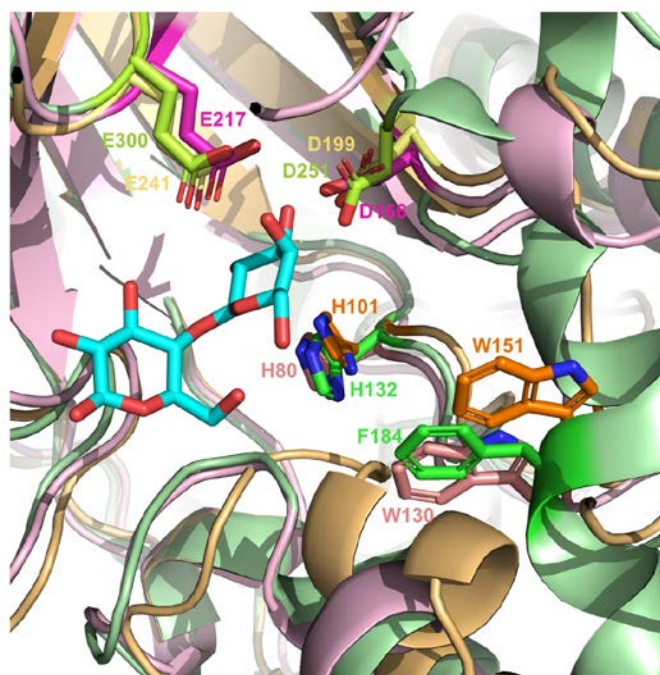
**Table S1.** Primers used in this study.

| Primer name           | Sequence (5'→3') <sup>a</sup>            |
|-----------------------|------------------------------------------|
| Fuc5372-WT forward    | 5'-CTGGGATCCCAAGATACCCTGCAAAATGAGACC     |
| Fuc5372-WT reverse    | 5'-ATTAAGCTTGGCTGCAGGTCGACCCTAGCTAGG     |
| Fuc5372-H101F forward | 5'CCACCAAAT <b>T</b> CCACGATGGTG         |
| Fuc5372-H101F reverse | 5'CACCATCGTG <b>GA</b> ATTTGGTGG         |
| Fuc5372-W151F forward | 5'CCCTGCTGGACT <b>TTTT</b> CACACCCTGAC   |
| Fuc5372-W151F reverse | 5'GGTGGCT <b>AA</b> AGTCCAGCAGGC         |
| Fuc5372-W151H forward | 5'CCCTGCTGGAC <b>C</b> ATTACACCCTGAC     |
| Fuc5372-W151H reverse | 5'GGTGGCT <b>AT</b> GGTCCAGCAGG          |
| Fuc5372-R230K forward | 5'CATCAACTCC <b>AAA</b> ATCCAAGGCTACGGCG |
| Fuc5372-R230K reverse | 5'CGCCGTAGCCTTGGAT <b>TTT</b> GGAGTTGATG |
| Fuc5372-Q242R forward | 5'CTACTCCTGAG <b>CGC</b> GGTGTCCCTG      |
| Fuc5372-Q242R reverse | 5'CAGGGACAC <b>CGC</b> GCTCAGGAGTAG      |
| Fuc2358-WT forward    | 5'-TTTTGCATGCATGATAAGCCTTGAAGAGATTG      |
| Fuc2358-WT reverse    | 5'-TTTTAAGCTTCTAAAAGCTGATGCGCAAAAC       |
| Fuc2358-H132F forward | 5'GTTGCGGAG <b>T</b> CCACGATGG           |
| Fuc2358-H132F reverse | 5'CCATCGTG <b>GA</b> ACTCCGCAAC          |
| Fuc2358-F184H forward | 5'CACCAGTT <b>T</b> CACTTTTTCACATG       |
| Fuc2358-F184H reverse | 5'CATGTGAAA <b>AGT</b> GAAACTGGTG        |
| Fuc2358-F184W forward | 5'CACCAGTT <b>T</b> GGTTTTTCACATG        |
| Fuc2358-F184W reverse | 5'CATGTGAAA <b>CC</b> AAACTGGTG          |
| Fuc2358-K286R forward | 5'GTTTGTTAC <b>CGC</b> CAGGATGCC         |
| Fuc2358-K286E reverse | 5'GGCATCCTG <b>CGC</b> GTAACAAAC         |
| Fuc2358-R301Q forward | 5'AGATGGAG <b>CAG</b> GGAGGCTACG         |
| Fuc2358-R301Q reverse | 5'CGTAGCCTCC <b>CTG</b> CTCCATCT         |

<sup>a</sup>Letters in bold indicate nucleotides that have been changed in the sequences of the genes that encode the wild-type enzymes to construct the mutant enzymes.

**Table S2.** Strains and plasmids used in this study.

| Strain or plasmid             | Relevant genotype or properties                      | Source                      |
|-------------------------------|------------------------------------------------------|-----------------------------|
| <b><i>E. coli</i> strains</b> |                                                      |                             |
| <b>DH10B</b>                  | <i>F-endA1 recA1 galE15 galK16 nupG rpsL</i>         | Invitrogen                  |
| <b>PE187</b>                  | DH10B containing pQE80fuc2358                        | Moya-González et al. (2022) |
| <b>E164</b>                   | DH10B containing pQE80fuc2358-R301Q                  | This work                   |
| <b>E165</b>                   | DH10B containing pQE80fuc2358-F184H                  | This work                   |
| <b>E166</b>                   | DH10B containing pQE80fuc2358-F184W                  | This work                   |
| <b>E167</b>                   | DH10B containing pQE80fuc2358-K286R                  | This work                   |
| <b>E168</b>                   | DH10B containing pQE80fuc2358-H132F                  | This work                   |
| <b>PE188</b>                  | DH10B containing pQE80fuc5372                        | Moya-González et al. (2022) |
| <b>E159</b>                   | DH10B containing pQE80fuc5372-H101F                  | This work                   |
| <b>E160</b>                   | DH10B containing pQE80fuc5372-Q242R                  | This work                   |
| <b>E161</b>                   | DH10B containing pQE80fuc5372-R230K                  | This work                   |
| <b>E162</b>                   | DH10B containing pQE80fuc5372-W151H                  | This work                   |
| <b>E163</b>                   | DH10B containing pQE80fuc5372-W151F                  | This work                   |
| <b>Plasmids</b>               |                                                      |                             |
| <b>pQE80</b>                  | <i>E. coli</i> expression vector; Amp <sup>R</sup>   | Qiagen                      |
| <b>pQE80fuc2358</b>           | pQE80 containing <i>fuc2358</i> coding region        | Moya-González et al. (2022) |
| <b>pQE80fuc2358-R301Q</b>     | pQE80 containing <i>fuc2358</i> -R301Q coding region | This work                   |
| <b>pQE80fuc2358-F184H</b>     | pQE80 containing <i>fuc2358</i> -F184H coding region | This work                   |
| <b>pQE80fuc2358-F184W</b>     | pQE80 containing <i>fuc2358</i> -F184W coding region | This work                   |
| <b>pQE80fuc2358-K286R</b>     | pQE80 containing <i>fuc2358</i> -K286R coding region | This work                   |
| <b>pQE80fuc2358-H132F</b>     | pQE80 containing <i>fuc2358</i> -H132F coding region | This work                   |
| <b>pQE80fuc5372</b>           | pQE80 containing <i>fuc5372</i> coding region        | Moya-González et al. (2022) |
| <b>pQE80fuc5372-H101F</b>     | pQE80 containing <i>fuc5372</i> -H101F coding region | This work                   |
| <b>pQE80fuc5372-Q242R</b>     | pQE80 containing <i>fuc5372</i> -Q242R coding region | This work                   |
| <b>pQE80fuc5372-R230K</b>     | pQE80 containing <i>fuc5372</i> -R230K coding region | This work                   |
| <b>pQE80fuc5372-W151H</b>     | pQE80 containing <i>fuc5372</i> -W151H coding region | This work                   |
| <b>pQE80fuc5372-W151F</b>     | pQE80 containing <i>fuc5372</i> -W151F coding region | This work                   |



**Fig. S1.** Structural alignment of Fuc2358 (pale green), Fuc5372 (light orange) and AlfB from *Lactocaseibacillus paracasei* (ex-*Lactobacillus casei*) structures obtained by AlphaFold2, indicating the catalytic residues (lime green for Fuc2358, yellow for Fuc5372 and magenta for AlfB). Amino acid residues subjected to point mutation are indicated in green (Fuc2358) orange (Fuc5372) and violet (AlfB).

|          |                         |                                        |              |     |
|----------|-------------------------|----------------------------------------|--------------|-----|
| FgFC01   | PEAIIRIVKSGSGARFAGPVAE  | HDGFSMWDSK-VN---EWNPV----              | NYGPKLDLVKLW | 179 |
| NixE     | PTGWAKLFRESGARYVVPVAE   | HDGFALYDSK-LS---DWTAM----              | KMGPKRDLLGEL | 218 |
| Fuc2358  | PASWDLDFQKAGAQYLFPAVE   | HDGFQMYAST-LS---PYNSL----              | EMGPRRDVLGEL | 161 |
| FoFC01   | AEDWMNLVADAGAQYVVPVTK   | HDGWALFDFP-ESV-SKRSTV----              | HYGPKRDFVKEL | 293 |
| DdFuc    | ANEWASIIKESGAKYVVLTSK   | HEGYTLWNSE-QSW-N-WNSV----              | ETGPGIDIVGEL | 158 |
| DmFuca   | ATKWALLFKDSGARYVVLTSK   | HDGFTLWPSK-NSY-G-WNSM----              | DVGPKRDIVKEL | 165 |
| PapFuC   | PKTFAEIIIEASGAKYVVLTSK  | HEGFTNWPSK-YSW-N-WNSM----              | DVGPKRDLVGDL | 163 |
| RnFucA1  | PEEWADLFQAAGAKYVVLTAH   | HEGFTNWPSA-VSW-N-WNSK----              | DVGPHRDVLGEL | 164 |
| ClFuc    | PDTWADLFQAAGARYVVLTTK   | HEGFTNWPSV-VSW-N-WNSN----              | DVGPHRDVLGEL | 168 |
| FucA1    | PEEWADLFQAAGAKYVVLTTK   | HEGFTNWPSV-VSW-N-WNSK----              | DVGPHRDVLGEL | 163 |
| Alfa     | VQAWAAAFANAGAKYVVIIVTK  | HDGFVMDPDH-TKN-P-YE-P----              | DYHLNDFVVGEL | 157 |
| TmaFuc   | PQEWADLFKAGAKYIIPVTTK   | HDGFCLWGTY-Y-T-D-FNSV----              | KRGPKRDVLGDL | 157 |
| Blon0248 | PADMADLFARAGARYFVPTTK   | HEGITLWKAP-DND-G-WNTV----              | DRGPHRDLVKEF | 159 |
| Blon0426 | PADMADLFARAGARYFVPTTK   | HEGITLWKAP-DND-G-WNTV----              | DRGPHRDLVKEF | 159 |
| Fuc35B   | TDQWIHTAKSLGAKYAVLVAKH  | CSGFSLWPTT-AHE---YSIK-NSPYKN-          | GKGDIVAEF    | 131 |
| BbAfcB   | TDQWMSFADGFGFKMIVTVTK   | HDGFLYDSR-YNTEHDWAT-AVAKRT-            | GKGLFRKI     | 612 |
| BT2192   | CRQWMQTLKAAGIPAAILTAKH  | ADGFCLWPSK-YTD---YSVK-NAAWKN-          | GKGDVVREF    | 131 |
| Fuc18    | CEQWVQTFVNSGMKGVIITAKH  | HDGFCLWPTQ-LTE---YCIR-NTPYKD-          | GKGDIVREL    | 133 |
| BiAfcB   | VDQWMDALVAGGMAGVILTCKH  | HDGFCLWPSR-LTR---HTVA-SSPWRE-          | GKGDLVREV    | 117 |
| BT1625   | AEQWVRTLKDAGFKMVLLTAKH  | HDGFCLWPTA-TTK---HSAV-SSPWKN-          | GQGDVVKEL    | 149 |
| Fuc39    | AEQWVRTLKDAGFKMVLLTAKH  | HDGFCLWPTA-TTK---HSAV-SSPWKN-          | GQGDVVKEL    | 150 |
| BT4136   | AEQWVSNLKEAGFKMVILTAKH  | HDGFCLWPTA-TTK---HSAV-SSWKN-           | GQGDVVKEL    | 149 |
| AtFUC1   | ASQWVQIAKDSGFSRVILTAKH  | HDGFCLWPSA-YTD---YSVK-SSQWRN-          | GAGDVVAEL    | 136 |
| CpAfc2   | ADQWVKTLADAGFGRVILTAKH  | HDGFCLWDSA-YTK---HDVA-SSPWKN-          | GKGDVVKEV    | 146 |
| SpGH29   | AREWVRVLKETGFKLILVVKH   | HDGFVLYPTA-HTD---YSVK-VSPWRR-          | GKGDLLLEV    | 109 |
| Fuc1584  | ADAIVSLAKEAGMRSIIITTKH  | HDGFCMRTA-TTD---YNSYDATPG----          | KRDFIKEM     | 139 |
| AlfC     | AKKIVALAKQCGMQLVVTTKH   | HDGFAMYHSK-VDA---YNNVDATPF----         | HRDIIGEL     | 117 |
| Fuc35A   | AKKWVRAAKHAGMKYLVYTTKH  | HDGFAMYNDA-CSD---YNIVKCTPF----         | AKDPLKEL     | 137 |
| Fuc30    | CKQWAKAASANMTYGCLTTKH   | HSGFCIWDTK-TTD---YSVM-SPFF----         | KRDVKEYE     | 121 |
| Fuc19A   | PAQWAEVTKQAGMKYIIFTTKH  | HDGFCMFDSQ-YTD---FSIS-RCAFKEHPKADVAKYV | 165          |     |
| AlfB     | AKKIAHAAKAVGAKYIVLTTKH  | HEGFFLYDTKGLSD---FDVM-HAPA----         | RRDLIAEF     | 110 |
| BT2970   | AKKWAKMAKEMGTKYVKITTKH  | HEGFCWPSK-YTK---YTVA-NTPY----          | KRDILGEL     | 164 |
| Fuc5372  | PKAWVDLIKESGAKYVITTKH   | HDGVALWDTK-VGD---ISVVKSTPA----         | KRDLIAPF     | 131 |
| TfFuc1   | AAEWVAAIKASGAKYITITSRH  | HDGFSMYATQ-QSD---YNNVDATPF----         | KRDVIEL      | 138 |
| Mfuc6    | PKEWARLAKRVGMQYAIFTSKH  | HDGYAMYHTA-END---FSIG-HSPY----         | GKDIVRSF     | 115 |
| Mfuc5    | PAEWAARAKAGMKYAVMTSKH   | HDGFCLFDSQ-YTD---YKAT-NTPA----         | GRDLVREY     | 107 |
| aLfuk1   | PKAWAKAARAKAGMKYAVMTSKH | HDGFCLFDSA-LTD---YKAT-NTPA----         | GRDLIREY     | 111 |
| Fuc193   | PEEWAYMAKAGVKYVMVFTTKH  | HDGFCMWDSK-YTD---YKIT-NTPY----         | KKDLTKPL     | 140 |
| Mfuc2    | PAAWARDARNAGMKYVVVTTKH  | HDGFCLWDSA-LTD---YKAP-NTPA----         | GRDLLRPL     | 113 |
| Mfuc7    | AGEWARQARDAGMKYVVMVTTK  | HEGFCMFDSR-YTD---YKCT-NTPA----         | GRDLVREY     | 114 |
| Mfuc1    | PRLWARAAREAGMKYVVLTTKH  | HEGFCWDSQ-FTD---YKVT-NTPY----          | GKDLLTPF     | 113 |
| Mfuc4    | PRKWARAAREAGMKYVVLTTKH  | HEGFCWDSA-YTD---YKAS-NTPY----          | GKDLLKPF     | 112 |

Fig. S2. Continues on the next page.

|          |                                                               |     |
|----------|---------------------------------------------------------------|-----|
| FgFC01   | ADLVRE--NDMKLVIAMHQAYNYNGFFQWA----PKTNDTS-LQKLL-----          | 219 |
| NixE     | STAIRA--QGLHFGLSHRAEHN--WFFDGGGRKFDSDVNDPR-YAALYGPAQ---VRLPGK | 271 |
| Fuc2358  | REEAEK--RGLHFCTSSHRAEHQ--FFFSGHKEFTSDISQEVPRNSLYWPAE---PEP--K | 213 |
| FoFC01   | LDVAKAKHPEIRRGTYFS-M--PEWFPNPAY----AKYYWDQHYKEIYWGRP---PTNPY- | 342 |
| DdFuc    | TKSVKN--MGLHMGLYHS-L--FEWFNPPLY-----LADAET----GKNP---T-----   | 194 |
| DmFuca   | AAAIRKE--SDLRFGLYYS-L--FEWFNPPLW-----TDDKLH----LL-----M-----  | 200 |
| PapFuC   | ATAIRNYTKDVHFGLYHS-L--FEWFNPPLY-----LQDKKG----GF-----S-----   | 199 |
| RnFucA1  | GA AVRK--RNIRYGLYHS-L--FEWFHPLY-----LLDKKN----GL-----K-----   | 198 |
| ClFuc    | GRALRK--RNIRYGLYHS-L--LEWFHPLY-----LLDKKN----NF-----K-----    | 202 |
| FucA1    | GTALRK--RNIRYGLYHS-L--LEWFHPLY-----LLDKKN----GF-----K-----    | 197 |
| Alfa     | AQAVRA--HGMRFGTYYSSL--LDWTFPHL----PIKDYGSFL----LGND---K-----  | 197 |
| TmaFuc   | AKAVRE--AGLRFGVYYSGG--LDWRFTE----PIRYPEDLS----YIRP---N-----   | 197 |
| Blon0248 | ADAMRD--KGLKFGVYYSSG--LDWHKEPN----MPIL--GDG---EYGP---Q-----   | 197 |
| Blon0426 | ADAMRD--KGLKFGVYYSSG--LDWHKEPN----MPIL--GDG---EYGP---Q-----   | 197 |
| Fuc35B   | VASCRK--YGIKPGIYAST--TANGFLHV---D-----NPGI-----NPGL           | 161 |
| BbAfcB   | VASAKK--YGLKVGIIYSP--ADSYMERK---GVWGNNSARVERTIPTLVENDDRAGK    | 663 |
| BT2192   | VDACEE--YGLKAGIYLG--HDRHEHLS---P-----                         | 157 |
| Fuc18    | SDACKK--YGIKFAVYLSP--WDRHQA-----                              | 156 |
| BiAfcB   | SESARR--HGLKFGVYLSP--WDRTEESY---G-----                        | 143 |
| BT1625   | RNACDK--YDMKFGVYLSP--WDRNAECY---G-----                        | 175 |
| Fuc39    | RNACDK--YDMKFGVYLSP--WDRNAECY---G-----                        | 176 |
| BT4136   | RKACKK--YGMRFGLYLSP--WDRNAECY---G-----                        | 175 |
| AtFUC1   | ASAAKE--AGIGLGLYLSP--WDRHEQCY---G-----                        | 162 |
| CpAfc2   | SEACAK--YNIKFGVYLSP--WDQNSEHY---G-----                        | 172 |
| SpGH29   | SQAATE--FDMDMGVYLSP--WDAHSPLY---H-----                        | 135 |
| Fuc1584  | AEACKR--GGINFGIYFSL--IDWHFPQA---YPISSHN-----CD                | 173 |
| AlfC     | AEACQK--AGLKFGLYYSQD--LDWHDPNG---GGYKSNDVETAGT---T---WDNSWD   | 163 |
| Fuc35A   | AEACKK--EGIKLGLYYSLG--RDWEDPDV---PTNWP-----VKA---G---RSNTWD   | 178 |
| Fuc30    | ADAFRA--EGMKVMLYYSI--LDTHARLR---PKC-----                      | 149 |
| Fuc19A   | FEAFRK--EGFMTGAYFSK--PDWHNQDY---WW--DYF---ATP---N---R---NVN   | 203 |
| AlfB     | VAACRE--EDLLPFFYMAT--YDWHTPLY---DD-----                       | 137 |
| BT2970   | VKAYND--EGIDVHFYFSV--MDWSNPDY---RY-----D                      | 192 |
| Fuc5372  | VKEVRK--QGLKLGFIYSL--LDWSHPDY---PNKTRT-----E                  | 163 |
| TfFuc1   | ADECRK--QGIRLHLYYSH--LDWRRDDY---YPLGRTG---KG-----             | 172 |
| Mfuc6    | LDAMRA--EGLRVGIYFSL--IDWHHPDY---PAFTDAD---RPY---R---WGQ---    | 154 |
| Mfuc5    | VDAFRA--EGLKVGFIYSL--LDWHHPDY---PIAGDSI---HPM---R---N---HPD   | 147 |
| aLfuk1   | ADAFRA--EGLKVGFIYSI--IDWHHPDY---PAYGDRQ---HPM---R---D---NAE   | 151 |
| Fuc193   | VNAFRN--EGIRIGFIYSL--LDWHHPDF---TI--DRN---HPQ---M---PQNPEL    | 180 |
| Mfuc2    | VEAFRA--EGLKIGFIYHSL--LDWHHPDF---PV--DGL---HPM---R---D---DLA  | 151 |
| Mfuc7    | ADAFRA--EGLRVGFIYSL--IDWHHPDF---PI--DML---HPR---R---E---DTD   | 152 |
| Mfuc1    | VEAFRA--EGIRVGFIYSI--IDWHHPEF---PV--DVF---HPL---R---D---HPD   | 151 |
| Mfuc4    | VEAFRA--EGLHVGFIYSI--IDWHHPDF---LI--DVF---HPL---R---N---HPE   | 150 |

**Fig. S2.** Continues on the next page.

|          |                                                                |     |
|----------|----------------------------------------------------------------|-----|
| FgFC01   | -----GQLPRDEEDQ-LWFDKHKREMLDH-----V-QPDIIWDF-----SLDSPGECGS    | 261 |
| NixE     | DDADVANDWTPVSQAWLD-DWLARTTELIDV-----Y-QPDLIYFDW-----WIAHPTFR-- | 319 |
| Fuc2358  | DHFDLT-SKPYPskeFLE-DWLLRTCELVRD-----Y-QPELLYFDW-----WIQHESFR-- | 260 |
| FoFC01   | TNKSIEYTGyVEVNDfINDIQNPQMEALFYD-----Y-DIEMLWCDI---GGPNKAPD--   | 391 |
| DdFuc    | -----TQVYVDEILMKQLKDIVTT-----Y-EPELIWADG---DWMQLSNY--          | 231 |
| DmFuca   | -----QQHFVERKVRPEQMELVQQ-----Y-LPEIIWSDG---DWEAPAKY--          | 237 |
| PapFuC   | -----TQTYTLDICLPHELHEIEN-----Y-KPDVLWSDG---DWEALPEY--          | 236 |
| RnFucA1  | -----TQHFVSTKTMPELYDLVNR-----Y-KPDLIWSDG---EWECPSY--           | 235 |
| ClFuc    | -----TQFFVRAKTMPELYDLVNR-----Y-EPDLIWSDG---EWKCPDTY--          | 239 |
| FucA1    | -----TQHFVSAKTMPELYDLVNS-----Y-KPDLIWSDG---EWECPTDY--          | 234 |
| AlfA     | -----SQTYKD-YVWHQWHELIDR-----Y-HPDVLWNDI---GYPDDHR--           | 232 |
| TmaFuc   | -----TYEYAD-YAYKQVMELVDL-----Y-LPDVLWNDM---GWPEKGKE--          | 233 |
| Blon0248 | -----SEDYAR-YMYSHVMDLIDE-----Y-QPSILWGD I---DVPKISEE--         | 233 |
| Blon0426 | -----SEDYAR-YMYSHVMDLIDE-----Y-QPSILWGD I---DVPKISEE--         | 233 |
| Fuc35B   | VKKGS----PVTQEEYNK-IVETQLTELWSN-----YGKLF EIWFDG---GVLSQ-----  | 203 |
| BbAfcB   | VASGKLPTFKYKATDYGA-YMLNQLYELLTE-----YGDISEVWFDG---AQGNT-----   | 709 |
| BT2192   | -----LYTTERYKE-YYAQLGELMSD-----YGKIETWWDG---AGADE-----         | 194 |
| Fuc18    | -----NYGTPEYVD-YFYKQLHELLTN-----YGDVFEIWF DG---ANGGDGWYGG      | 198 |
| BiAfcB   | -----K--GKAYDD-FYVGQLTELLTQ-----YGP IFSVWLDG---ANGEG-----      | 178 |
| BT1625   | -----D--SPKYNE-FFIRQLTELLTN-----YGEVHEVWFDG---ANGEG-----       | 210 |
| Fuc39    | -----D--SPKYNE-FFIRQLTELLTN-----YGEVHEVWFDG---ANGEG-----       | 211 |
| BT4136   | -----D--SPRYNK-FFIRQLTELLTN-----YGEVHEVWFDG---ANGEG-----       | 210 |
| AtFUC1   | -----K--TLEYNE-FYLSQMTPELLTK-----YGEIKEVWLDG---AKGDG-----      | 197 |
| CpAfc2   | -----EGNGGDYNE-FYMNQLRELLTN-----YGP IAEVWMDG---AKGSN-----      | 209 |
| SpGH29   | -----VDREADYNA-YYLAQLKEILSNPNYGNAGKFAEVWMDG---ARGEG-----       | 177 |
| Fuc1584  | F----ITP-----QHHE-FTKAQVTELLTN-----YGP ISELWFD M---GS-----     | 206 |
| AlfC     | F---PDEDQKNFDLCFDN-KILPQIKEIMSN-----YGD IATAWFDV---PM-----     | 203 |
| Fuc35A   | Y---PDEDSKQLPAYIER-KVKPQLKELLTN-----YGE IAVIWFDT---PE-----     | 218 |
| Fuc30    | -----ITPQHIE-MIKEQLRELLTN-----YGEIT ALIIDGWDAPWSRIS----        | 188 |
| Fuc19A   | Y---KIERHPEKWEAFKT-YTHNQIGELMSN-----YGTIDILWLDG---GWVSPR----   | 247 |
| AlfB     | -----DFPAYLT-YLQKSVEVLCRN-----YGPVGGFWFDG---NWNK-----          | 171 |
| BT2970   | I---KSKEDSIAFSRfLE-FTDNQLKELATR-----YPTVKDFWFDG---TWDASV----   | 236 |
| Fuc5372  | V---RYKNDPERWARFNK-FNFGQLAELNKT-----W-KPDLYWFDG---DWEQ-----    | 204 |
| TfFuc1   | -----TGRTTQGKWEDYCA-FMNNQLTELLTN-----YGLIGAIWFDG---MWDKDI-YP-  | 217 |
| Mfuc6    | ----WRRSSPEAWVRYQQ-FMFGQIRELLTE-----YGKIDIIWFDG---GWER-----    | 195 |
| Mfuc5    | F---Q--NHTGNLPRYAD-YVRDQVRELLTN-----YGMIDIIWFD F---SYGEMR-GE-  | 191 |
| aLfuk1   | F---K--DRPQDFNRYLD-YMHGQVKELLTN-----YGTIDVLWFD F---SYEDMT-GE-  | 195 |
| Fuc193   | L---KELNKNRNMAYRE-YMKNQLTELLTE-----FGQIDELFMDY---TYAE-----     | 222 |
| Mfuc2    | F---REAAQGRDMARYRE-YLFGQTRELLTQ-----FGKIDIMWFD F---SYSELN-WG-  | 197 |
| Mfuc7    | A---REMSHGRDIRKYAC-YMRDQVRELLTN-----YGKIDILWFD F---SYGSWT-GEG  | 199 |
| Mfuc1    | V---AEINQGRDVRRYAE-YLRNQVEELLTR-----YN-PEILWCD F---SYPHAT-YK-  | 196 |
| Mfuc4    | V---ARLNEGRDMRRYAA-YMRNQVTELLTN-----YGDIDIIWFD F---SYPGRD-YN-  | 196 |

Fig. S2. Continues on the next page.

|          |                                                            |     |
|----------|------------------------------------------------------------|-----|
| FgFC01   | -----FEGPCAVDEQKRLEFLAYYFNERGE---EWGKEVVTYKHHD-----        | 299 |
| NixE     | -----SSLPTMLAYYYNQGAARTEADRGVVVNYKLG-----                  | 350 |
| Fuc2358  | -----PYLMRFLAYYYNLAA---QEDRKVAVCYKQD-----                  | 288 |
| FoFC01   | -----VL---APWLNWARDQGR-----QVTFNDRCCGA-----                | 415 |
| DdFuc    | -----WK---STEFLSWLYTNSSVKD---TVIVNDRWGSEC---RD-----        | 263 |
| DmFuca   | -----WR---SEEFIAWLYNDSPVRD---TVVTNDRWGFGT---AC-----        | 269 |
| PapFuC   | -----WN---STEFALWLCNDSPVKD---TVVTNDRWGQGT---SC-----        | 268 |
| RnFucA1  | -----WN---STEFALWLYNESPVKD---QVVVNDRWGQNC---SC-----        | 267 |
| ClFuc    | -----WN---STEFLSWLYNDSPVKD---HVVVNDRWGQNC---SC-----        | 271 |
| FucA1    | -----WN---STNFLSWLYNDSPVKD---EVVVNDRWGQNC---SC-----        | 266 |
| Alfa     | -----LETLFKYYYQQVP-----EGLVNDRWQQFP---DWMRTSW              | 264 |
| TmaFuc   | -----DL---KYLFAYYYNKHP-----EGSVNDRWGV-----                 | 257 |
| Blon0248 | -----DNDFSVARLFEHYDVVP-----DGVVNDRWGL-----                 | 261 |
| Blon0426 | -----DNDFSVARLFEHYDVVP-----DGVVNDRWGL-----                 | 261 |
| Fuc35B   | -----QNG-----GADILTLIQLRP-----NSIAFQGPYGY-PNLRWVGNEE       | 241 |
| BbAfcB   | -----AGTEHYD---YGVFYEMIRRLQP-----QAIQ-ANAA---YDARWVGNEE    | 747 |
| BT2192   | -----LTPPV---YRHWHYKIVREKQP-----DCVIFGTKNSYPFADVRWVGNEA    | 235 |
| Fuc18    | AKDARTIDRKTYD---YPRAYKMIDELQP-----QAVIFSDGG---PGCRWVGNEE   | 244 |
| BiAfcB   | -----KNGKTQYYD---WDRYNNVIRSLQP-----DAVI-SVCG---PDVRWAGNEA  | 218 |
| BT1625   | -----PNGKKQIYD---WDAFYKTIQQLQP-----KAVM-AIMG---DDVRWVGNEK  | 250 |
| Fuc39    | -----PNGKKQIYD---WDAFYKTIQQLQP-----KAVM-AIMG---DDVRWVGNEK  | 251 |
| BT4136   | -----PNGKKQVYD---WDTVYETIHLRP-----KAVM-AIMG---DDIRWVGNEE   | 250 |
| AtFUC1   | -----EKDMEYF---FDTWFSLIHQQLQP-----KAVIFSDAG---PDVRWIGDEA   | 236 |
| CpAfc2   | -----V-KQEYN---FEEWFALIKELQP-----ECLIFSPQG---PDIRWIGNEK    | 247 |
| SpGH29   | -----AQKVNYE---FEKWFETIRDQLG-----DCLIFSTEG---TSIRWIGNER    | 216 |
| Fuc1584  | -----N-TPEQ---SKELYQLVHRLQP-----DCMVSGRLLGNDQ-----         | 236 |
| AlfC     | -----TLSEAQ---SQTIYDTVRELQP-----NCLINSRLGNGK-----          | 234 |
| Fuc35A   | -----LVTRQQ---SKELRELIHSLQP-----GCLINSRIGNGL-----          | 249 |
| Fuc30    | -----YDDVP---FEDIYRLVKSIQP-----NCLVMDLNAAKY---PA-----      | 220 |
| Fuc19A   | -----NNQDID---MPQIAAMARAKQP-----GLLVVDRTIHGK-----          | 278 |
| AlfB     | -----KDADWH---LPELYGMIRHYQP-----NAIIVNNTGLKN---RGQVSD-     | 208 |
| BT2970   | -----KKNQW---TAHAEQMLKELVP-----GVAINSLRADD---KGKRHFD       | 274 |
| Fuc5372  | -----SAEAWN---SKDIVDLLRSDNK-----NVIINSIQGY-----            | 234 |
| TfFuc1   | -----DGMTAKTWN---LNEQYTLIHLRP-----ACLIGNNHHTP---FA-----    | 253 |
| Mfuc6    | -----TPDEWK---ATELEAMIRSLQP-----EILINDRLPGA-----           | 225 |
| Mfuc5    | -----AWK---ATELVKMVRELQP-----GIIIDNRLGGDI---KAAEPE-        | 225 |
| aLfuk1   | -----KWK---ATELVKMIRELQP-----NVLIDNRLGGNI---KAREPE-        | 229 |
| Fuc193   | ---GENGKNSKDWD---AEGIVKLARKLQP-----QIIINNRGLTE---NR-----   | 260 |
| Mfuc2    | ---WSKGRGKADWH---SEELMAMVRELQP-----GILLNDRLEVG-----        | 232 |
| Mfuc7    | DKAWMKGKGKDDWE---SEELIRIARELQP-----GIIIDNRTEIE-----        | 237 |
| Mfuc1    | ---GLPGKGRNDWQ---SESLVAMIRAIISP-----KIILNRLDLPI---A-----   | 233 |
| Mfuc4    | ---GLPGKGRNEWEE---SEKLYALVRRLRP-----HIIIDNRLDLPA---HL----- | 234 |

**Fig. S2.** Continues on the next page.



|          |                                                 |     |
|----------|-------------------------------------------------|-----|
| FgFCO1   | -----HG-----FRNTSAV-----DDWER-----              | 313 |
| NixE     | -----A-----FPEGAGT-----LDIER-----               | 363 |
| Fuc2358  | -----A-----LPFGSGI-----VEMER-----               | 301 |
| FoFCO1   | -----AGDYST-----PEYA-----                       | 425 |
| DdFuc    | -----KNGGFYT-----GADH-----                      | 274 |
| DmFuca   | -----MHGDFYN-----CADR-----                      | 280 |
| PapFuC   | -----KHGGFYS-----CNDR-----                      | 279 |
| RnFucA1  | -----RHGGYYN-----CEDK-----                      | 278 |
| ClFuc    | -----HHGGYYN-----CQDK-----                      | 282 |
| FucA1    | -----HHGGYYN-----CEDK-----                      | 277 |
| AlfA     | IRPIFNLVAAQVIKRDQHSNDLSEVKYYDYRT-----FEYRT----- | 302 |
| TmaFuc   | -----PHWDFKT-----AEYHM-----                     | 269 |
| Blon0248 | -----THWDFRT-----VEYEQ-----                     | 273 |
| Blon0426 | -----THWDFRT-----VEYEQ-----                     | 273 |
| Fuc35B   | -----GNSPYPCWATADATTSADGVQKI-----               | 264 |
| BbAfcB   | -----GWARQTEWSPQAAYNDGVQKV-----SLKPG-----       | 773 |
| BT2192   | -----GEAGDPCWATTDVAIRDEAQYY-----                | 258 |
| Fuc18    | -----GFAGATNWSFLRGGEVYPGYPKY-----               | 267 |
| BiAfcB   | -----GHVRDNEWSVVPRLRSALTEKSSQEDDASFATT-----     | 254 |
| BT1625   | -----GLGRETEWSATVLTGP-----IYARSEENNKRLG-----    | 279 |
| Fuc39    | -----GLGRETEWSATVLTGP-----IYARSEENNKRLG-----    | 280 |
| BT4136   | -----GLGRETEWSTTVLTPE-----IYARADKNNKRLG-----    | 279 |
| AtFUC1   | -----GLAGSTCWSLFNRTNAKIGDTEP-----               | 259 |
| CpAfc2   | -----GYAGEPCWSTIDIEKMK-----ERENP-----           | 269 |
| SpGH29   | -----GYAGDPLWQKVNPDKLG-----TEAEL-----           | 238 |
| Fuc1584  | -----YDFSV-----MADNTY-----PEGSL-----            | 252 |
| AlfC     | -----YDFVS-----LGDNEI-----PKNKED-----           | 251 |
| Fuc35A   | -----GDYSI-----IEQKLS-----NS-----               | 262 |
| Fuc30    | -----EALFYTDIKS-----YEQGAG-----QHIS-----        | 240 |
| Fuc19A   | -----YENYQT-----PEQKIP-----EHQ-----             | 293 |
| AlfB     | -----PEIDVVT-----YERRTP-----DEIYHG-----         | 227 |
| BT2970   | -----SNGRLMGDYES-----GYERRLP-----DPVK-----      | 296 |
| Fuc5372  | -----GDYAT-----PEQGV-----VVRP-----              | 249 |
| TfFuc1   | -----GEDIQI-----FERDLP-----GENKAG-----          | 271 |
| Mfuc6    | -----GDYDT-----PEQFVP-----PQLP-----             | 240 |
| Mfuc5    | -----IYAGDFAS-----PEQIIP-----PEGV-----          | 243 |
| aLfuk1   | -----IYAGDFAS-----PEQLLP-----PHGI-----          | 247 |
| Fuc193   | -----QGWDYIT-----PGQFMP-----QQWP-----           | 277 |
| Mfuc2    | -----GDIVT-----PEQYQP-----HGWM-----             | 247 |
| Mfuc7    | -----QDLWT-----PEQYQP-----TEWV-----             | 252 |
| Mfuc1    | -----ADIYT-----PEQVQP-----TEWV-----             | 248 |
| Mfuc4    | -----ADVHT-----PEQWQP-----TEWV-----             | 249 |

**Fig. S2.** Multiple sequence alignment (MSA) of characterized GH29A and GH29B  $\alpha$ -L-fucosidases listed in CAZy and  $\alpha$ -L-fucosidases previously isolated from the bacterial intestinal metagenome by our group<sup>1</sup>, using Clustal Omega<sup>2</sup>. Residue numbering for each sequence is indicated on the right side of the alignment. Nucleophile, acid-base and residues selected for mutations in Fuc2358 and Fuc5372 are highlighted, representing residues that are structural homologs in both enzymes based on AlphaFold2 analysis in the same color (H101 from Fuc5372 and H132 from Fuc2358, orange; W151 from Fuc5372 and F184 from Fuc2358, purple; D199 in Fuc5372 and D251 in Fuc2358, green; R230 in Fuc5372 and K286 in Fuc2358, pink; E241 in Fuc5372 and E300 in Fuc2358, red; Q242 in Fuc5372 and R301 in Fuc2358, blue). Residues sequence homologous to the residues selected for mutations in Fuc2358 and Fuc5372 are framed in the same black box. Residues that are conserved in all sequences in the alignment are indicated with “\*” below sequences; conserved substitutions are indicated with “.”; semi-conserved substitutions are represented with “.”. FgFCO1: *Fusarium graminearum* AFR68935.1; NixE: *Xanthomonas campestris* pv. *campestris* AAM42160.1; Fuc2358: uncultured *Streptococcus* sp. UUB87428.1; FoFCO1: *Fusarium oxysporum* AFR68934.1; DdFuc: *Dictyostelium discoideum* AAO51149.1; DmFuca: *Drosophila melanogaster* AAM50292.1; PapFuC: *Patiria pectinifera* BBG92283.1; RnFucA1: *Rattus norvegicus* CAA34268.1; ClFuc: *Canis lupus familiaris* CAA63362.1; FucA1: *Homo sapiens* AAA52481.1; AlfA: *Lactobacillus casei* CAQ67115.1; TmaFuc: *Thermotoga maritima* TAAD35394.1; Blon0248: *Bifidobacterium longum* subsp. *infantis* ACJ51376.1; Blon0426: *Bifidobacterium longum* subsp. *infantis* ACJ51546.1; Fuc35B: uncultured *Bacteroides* sp. UUB87424.1; BbAfcB: *Bifidobacterium bifidum* BAH80310.1; BT2192: *Bacteroides thetaiotaomicron* AAO77299.1; Fuc18: uncultured *Bacteroides* sp. UUB87420.1; BiAfcB: *Bifidobacterium longum* subsp. *infantis* ACJ53394.1; BT1625: *Bacteroides thetaiotaomicron* AAO76732.1; Fuc39: uncultured *Bacteroides* sp. UUB87425.1; BT4136: *Bacteroides thetaiotaomicron* AAO79241.1; AtFUC1: *Arabidopsis thaliana* NP\_180377.2; CpAfc2: *Clostridium perfringens* ABG83106.1; SpGH29: *Streptococcus pneumoniae* AAK76203.1; Fuc1584: uncultured *Phocaeicola* sp. UUB87427.1; AlfC: *Lactobacillus casei* CAQ67984.1; Fuc35A: uncultured *Bacteroides* sp. UUB87423.1; Fuc30: uncultured *Bacteroides* sp. UUB87422.1; Fuc19A, uncultured *Bacteroides* sp. UUB87421.1; AlfB: *Lactobacillus casei* CAQ67877.1; BT2970: *Bacteroides thetaiotaomicron* AAO78076.1; Fuc5372: uncultured *Phocaeicola* sp. UUB87429.1; TfFuc1: *Tannerella forsythia* AEW21393.1; Mfuc6:

uncultured bacterium AIC77303.1; Mfuc5: uncultured bacterium AIC77302.1; ALfuk1: *Paenibacillus thiaminolyticus* CBM40947.1; Fuc193: uncultured *Bacteroides* sp. UUB87426.1; Mfuc2: uncultured bacterium AIC77299.1; Mfuc7: uncultured bacterium AIC77304.1; Mfuc1: uncultured bacterium AIC77298.1; Mfuc4: uncultured bacterium AIC77301.1.

### Supplementary material references

1. Moya-González EM, Peña-Gil N, Rubio-del-Campo A, *et al.* Infant gut microbial metagenome mining of  $\alpha$ -L-fucosidases with activity on fucosylated human milk oligosaccharides and glycoconjugates. *Microbiol Spectr.* 2022;10(4):e0177522. doi:10.1128/spectrum.01775-22
2. Sievers F, Wilm A, Dineen D, *et al.* Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol.* 2011;7:539. doi:10.1038/msb.2011.75