

2020 Ching Hou Biotechnology Award

**Bioprocess development for
novel functional lipid production:
screening and analysis of
microbial metabolisms and enzymes**

Jun Ogawa

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Division of Applied Life Sciences,
Graduate School of Agriculture,
Kyoto University
Kyoto, Japan**

Bioprocess development for novel functional lipid production: screening and analysis of microbial metabolisms and enzymes

- 1) Polyunsaturated fatty acid production and modification**
- 2) Gut microbial fatty acid metabolism**

1) Polyunsaturated fatty acid production and modification

Various physiological activity

- Cardiovascular fitness
- Cerebral function development
- Anticancer
- Lipid metabolism control
- Immune and inflammation control

Precursor of active metabolites

- prostaglandin, leukotriene, thromboxane
- Anti-inflammatory mediators: resolvins etc.

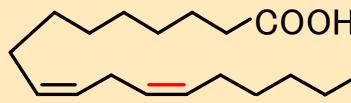
Current source...fish oil, plant oil

- unstable supply
- low purity
- contamination of unhealthy compounds

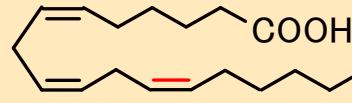


**Microbial oil
as alternative source**

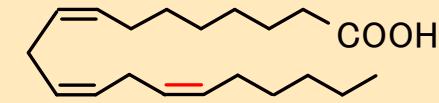
$\omega 6$ (n-6) PUFAs



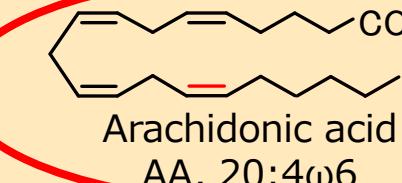
Linoleic acid
LA, 18:2 ω 6



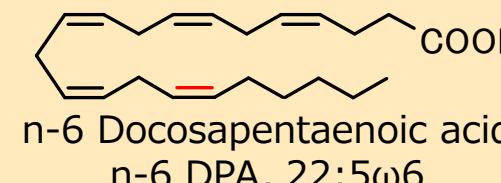
γ -Linolenic acid
GLA, 18:3 ω 6



Dihomo- γ -linolenic acid
DGLA, 20:3 ω 6

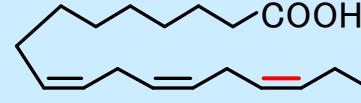


Arachidonic acid
AA, 20:4 ω 6

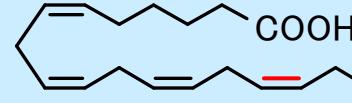


n-6 Docosapentaenoic acid
n-6 DPA, 22:5 ω 6

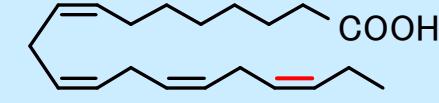
$\omega 3$ (n-3) PUFAs



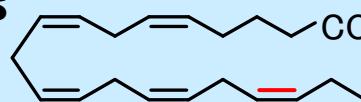
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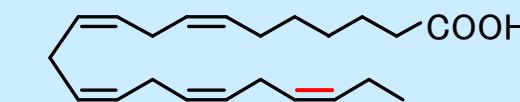
Stearidonic acid
SDA, 18:4 ω 3



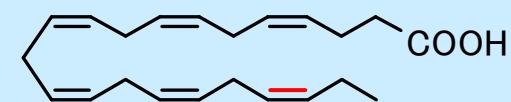
Eicosatetraenoic acid
ETA, 20:4 ω 3



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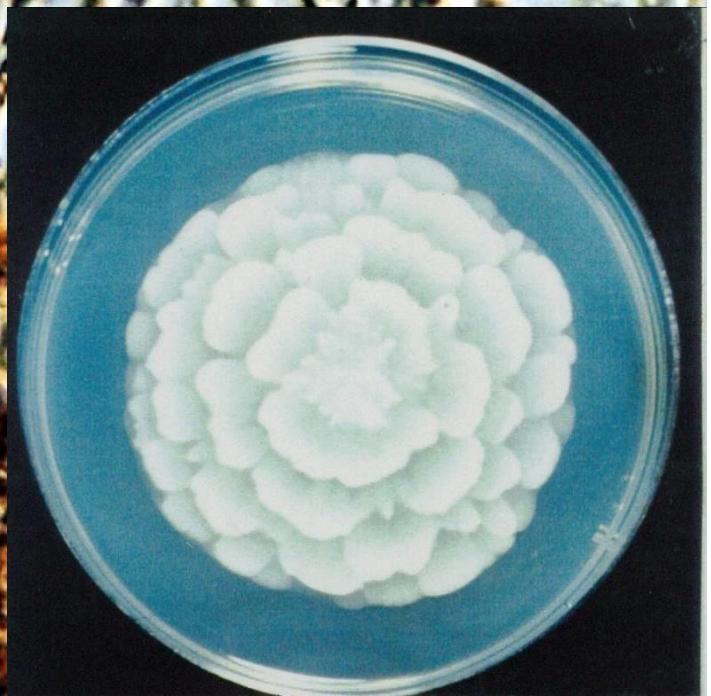
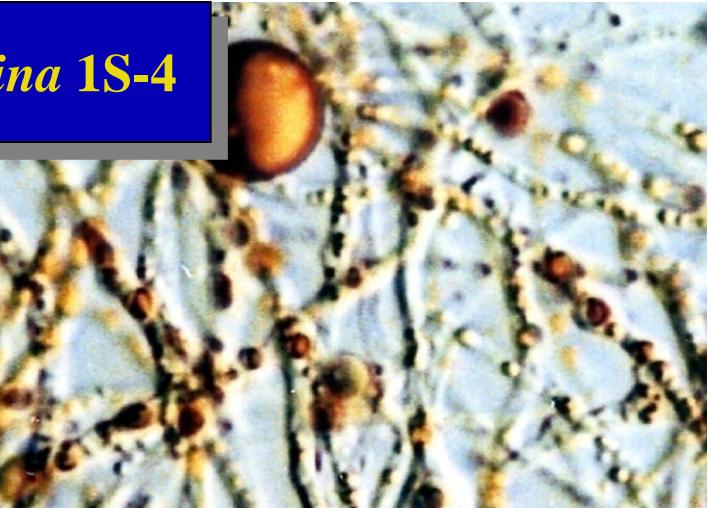


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Docosahexaenoic acid
DHA, 22:6 ω 3

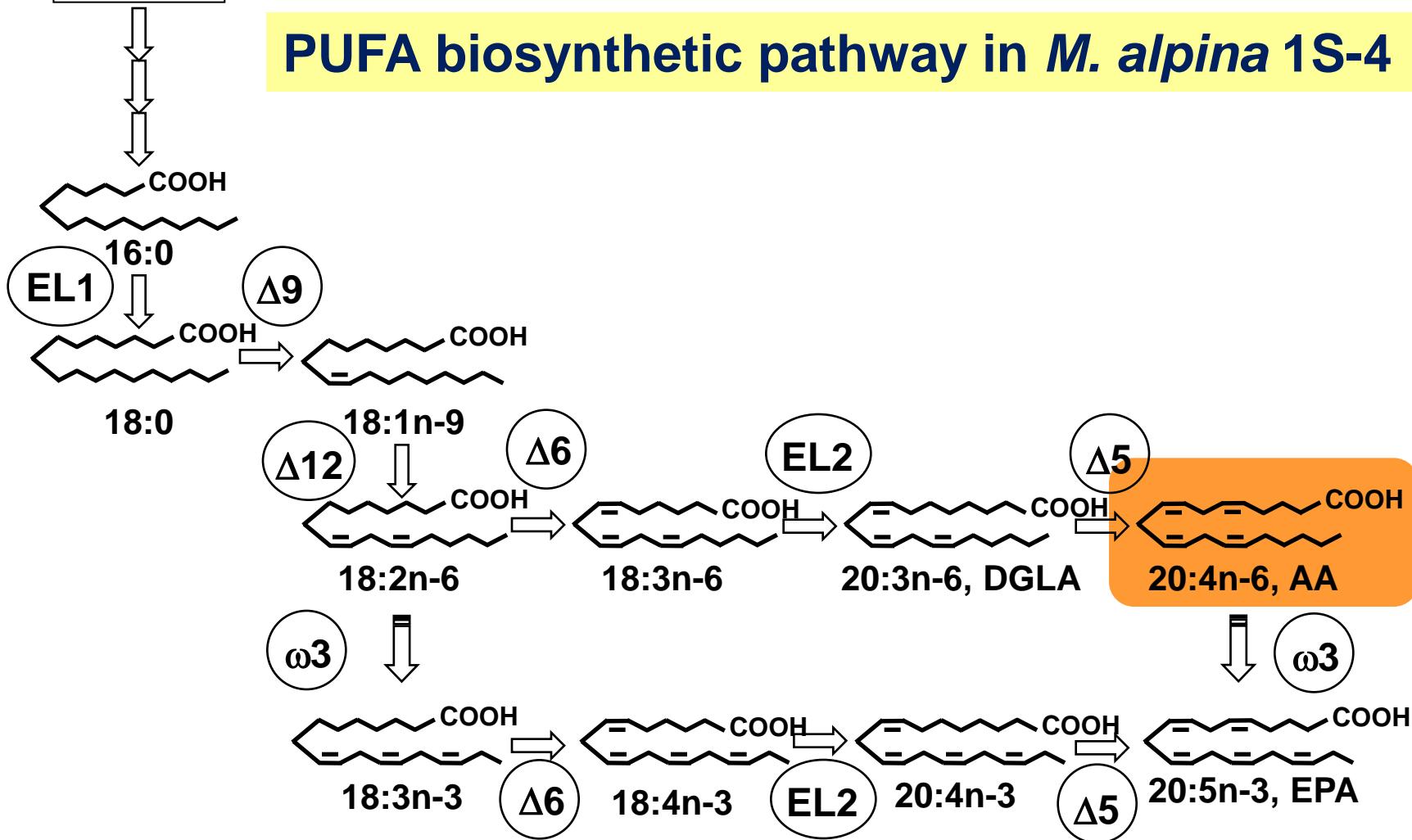
Microscopic observation of *Mortierella alpina* 1S-4



- Lipid productivity (**600 mg/g** dry mycelia)
- Lipid bodies containing **triacylglycerol rich in arachidonic acid** in fungal filament

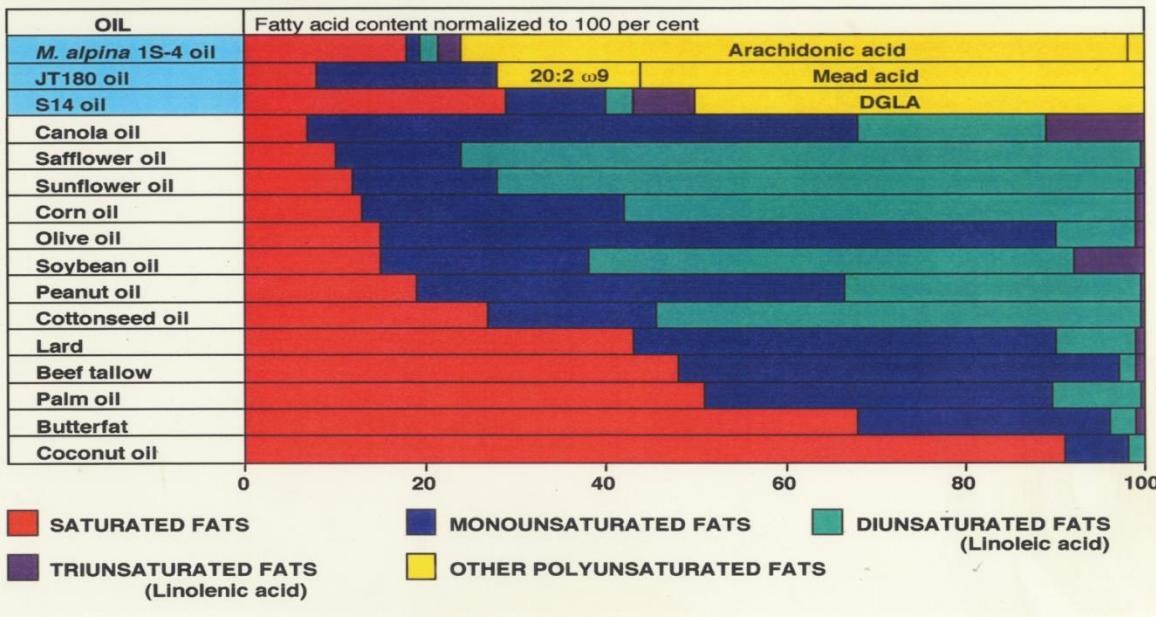
Glucose

PUFA biosynthetic pathway in *M. alpina* 1S-4

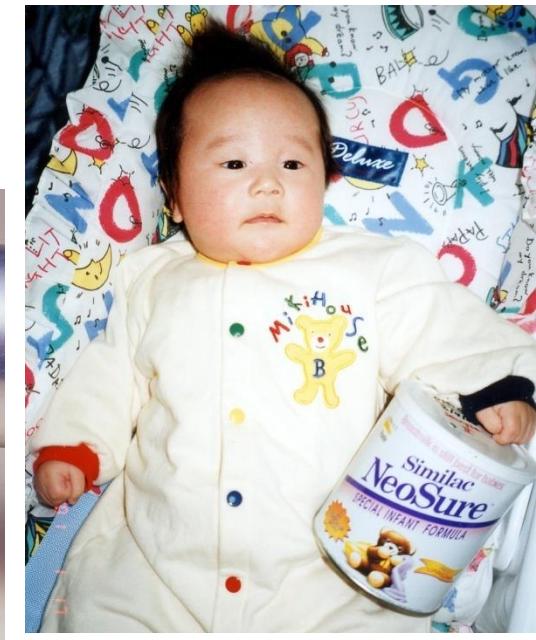


Fatty acid profile of “Single Cell Oil” produced by *M. alpina* is quite different from common edible oils and is used as an ingredient for infant formula in the world.

Comparison of Microbial Oils with Dietary Fats

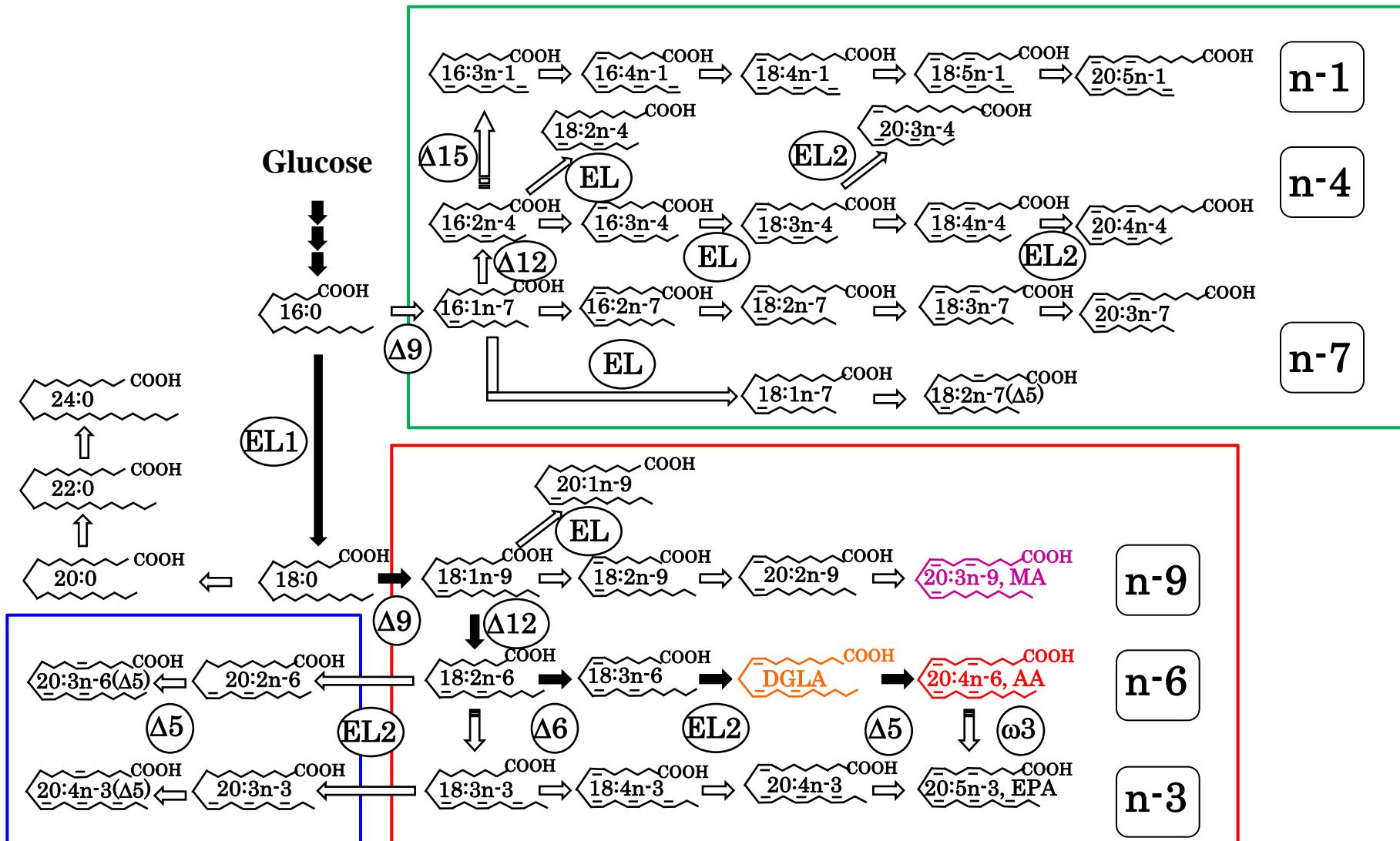


Used for
Infant milk
ingredients



Looks like plant oils for diet,
but the fatty acid profile is
quite different and
rich C20 n-6 PUFA

Various PUFA biosynthetic pathways in *M. alpina* and its mutants



1) Polyunsaturated fatty acid production and modification

Various physiological activity

- Cardiovascular fitness (Medical use)
- Cerebral function development
- Anticancer
- Lipid metabolism control
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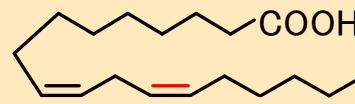


Microbial oil

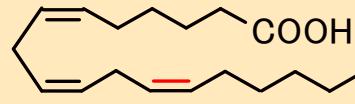
as alternative source

Molecular breeding for efficient production

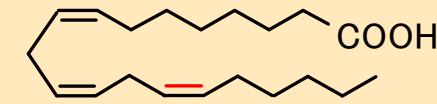
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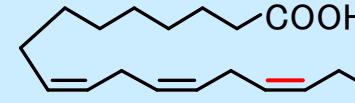


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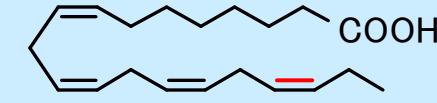


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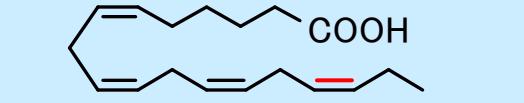
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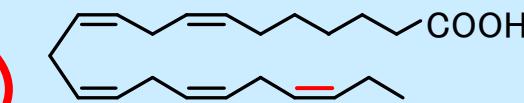
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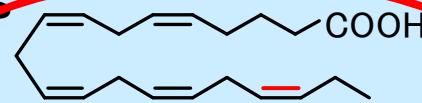
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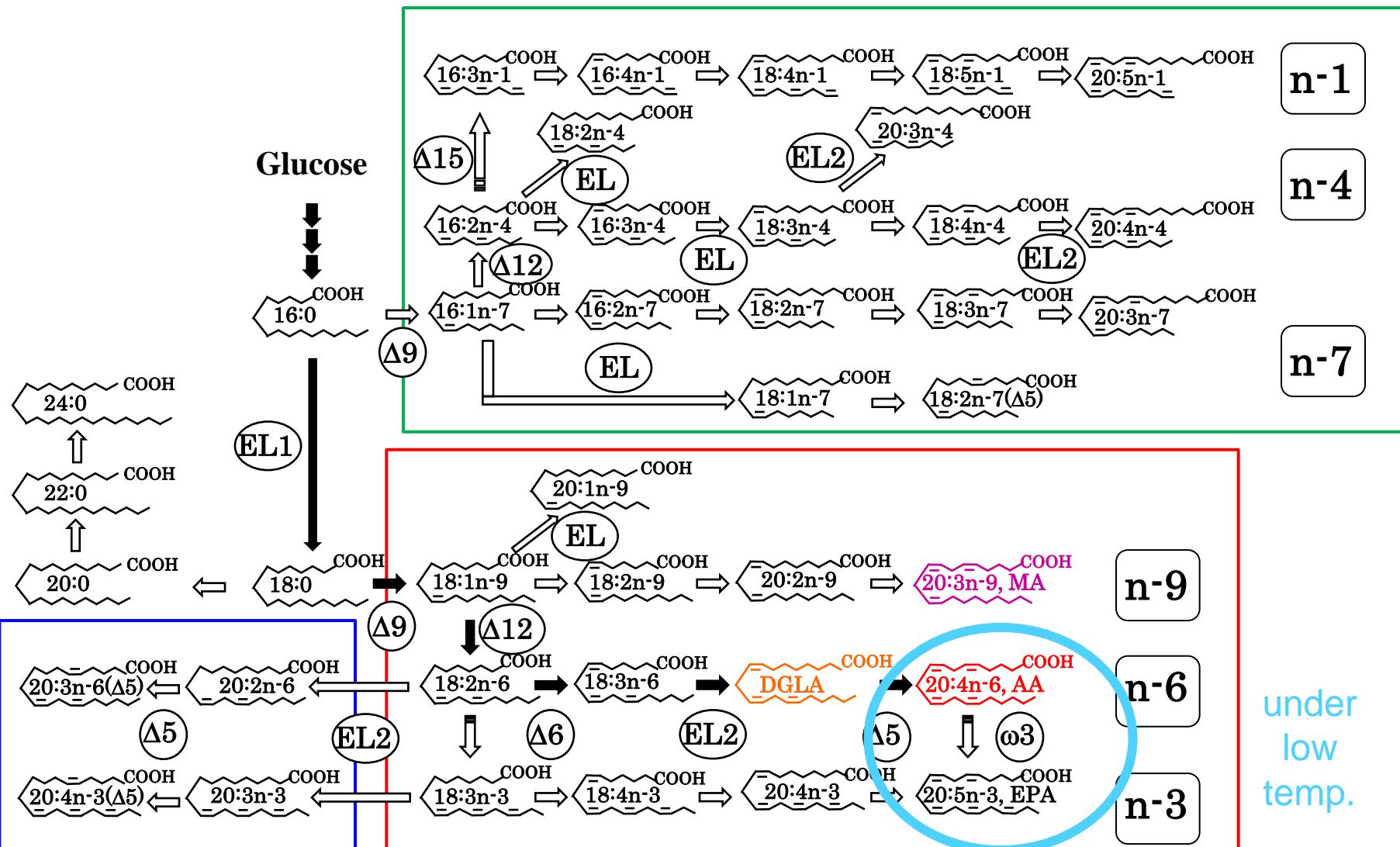


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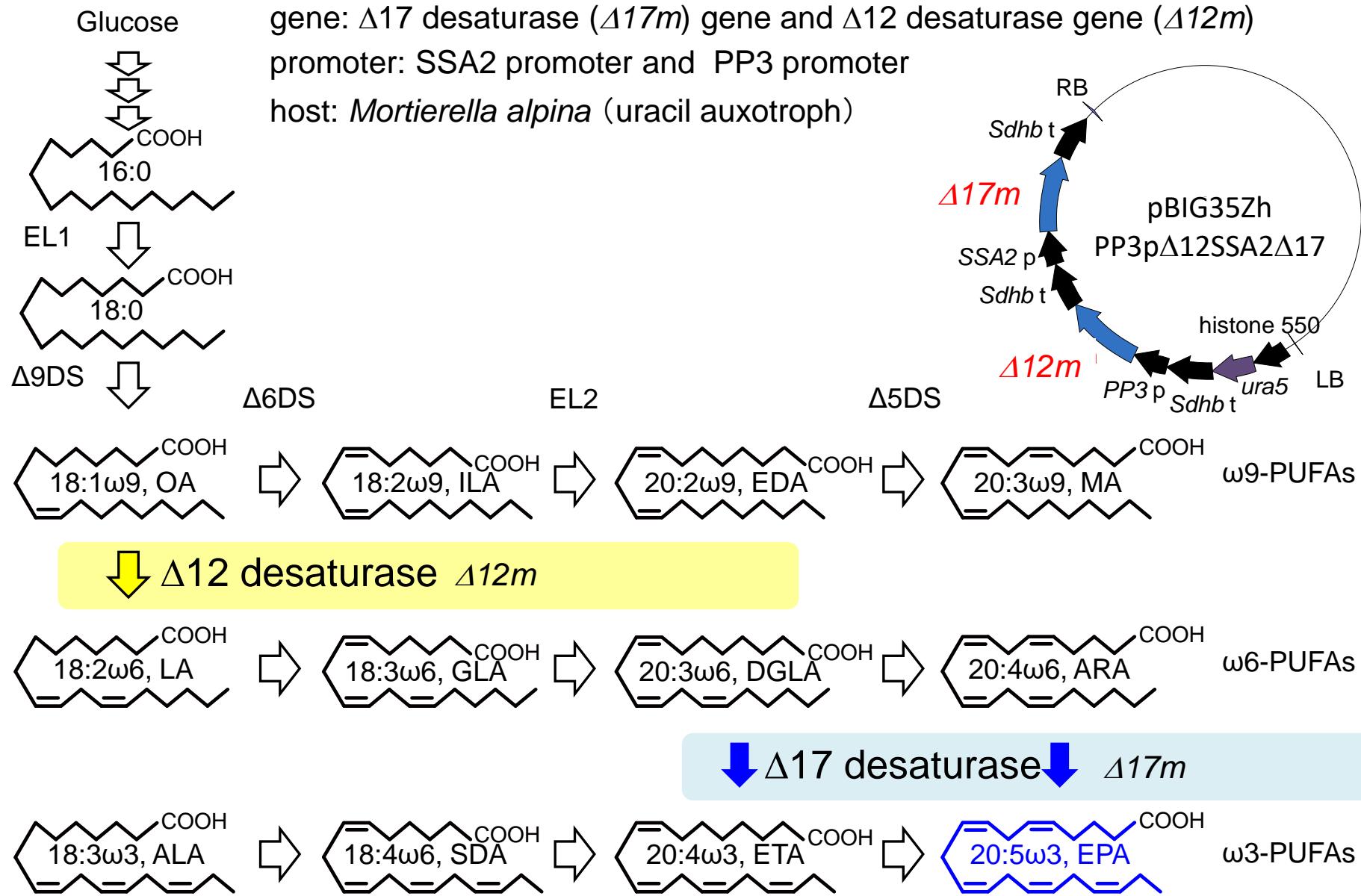
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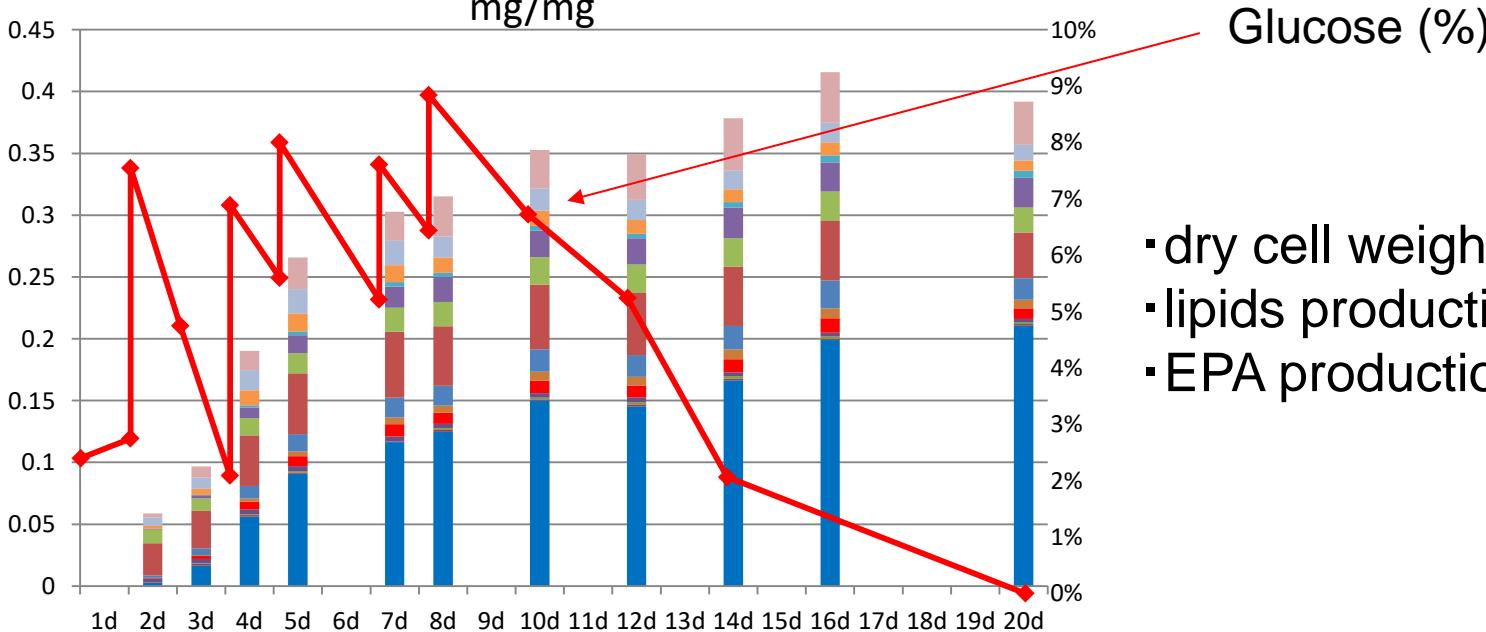
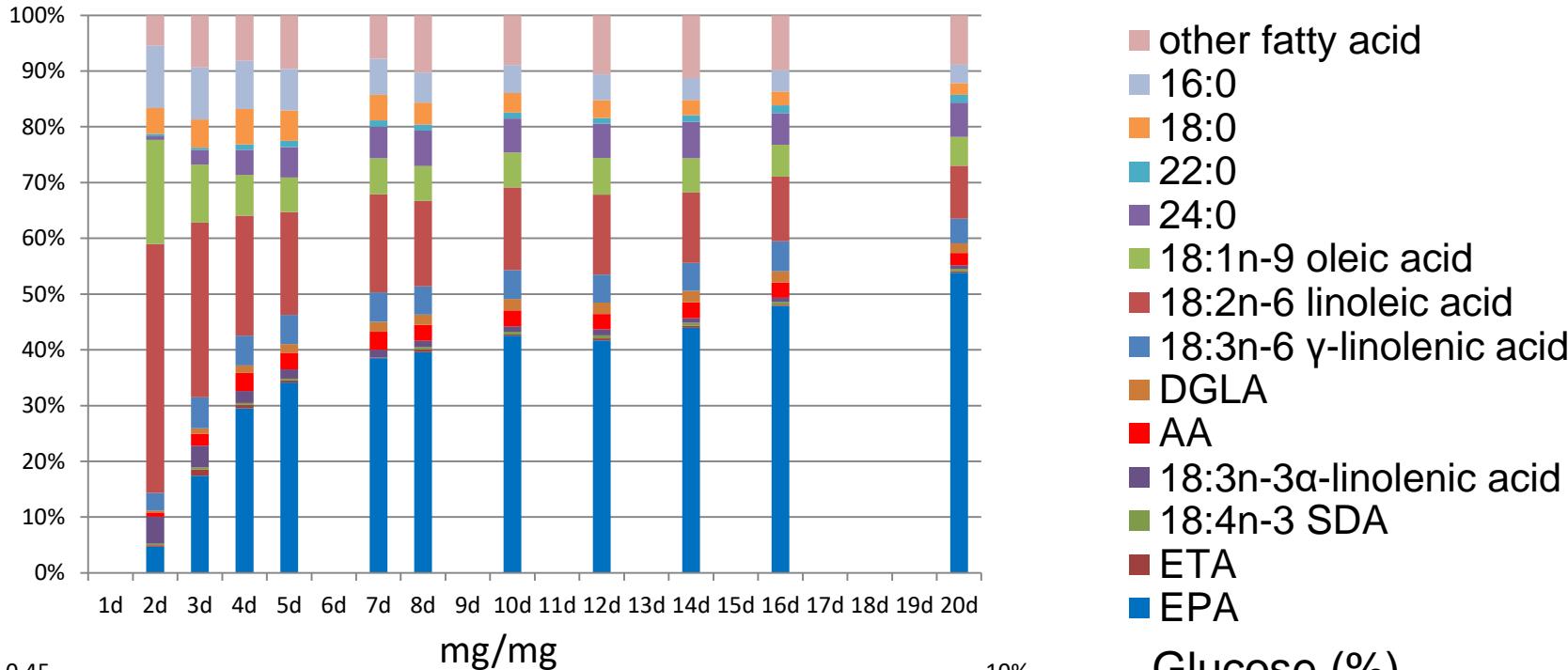


EPA production by expression of heterologous desaturase genes
in *M. alpina* 1S-4 under ordinary temperature

EPA production by expression of heterologous desaturase genes in *M. alpina* 1S-4 under ordinary temperature



5L jar fermentor scale EPA production (26°C, 20 days cultivation)



- dry cell weight 70 g/L
- lipids production 28 g/L
- EPA production 52 % 14.4g/L

1) Polyunsaturated fatty acid production and modification

Various physiological activity

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- Anticancer
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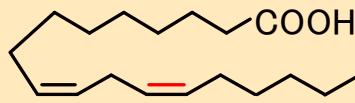
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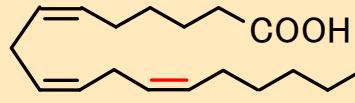


**Microbial oil
as alternative source
Production of rare PUFAs
for future development**

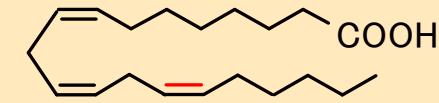
$\omega 6$ (n-6) PUFAs



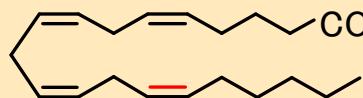
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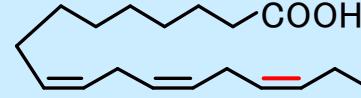


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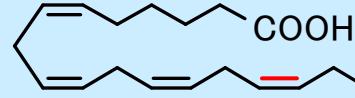


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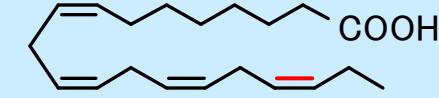
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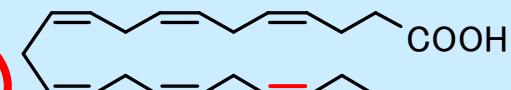
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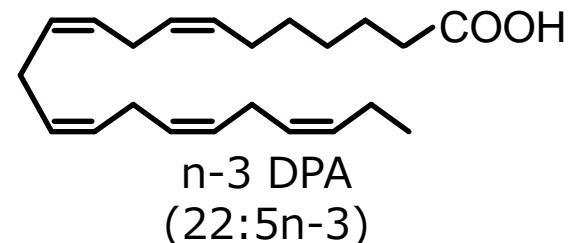


Docosahexaenoic acid
DHA, 22:6 ω 3

Introduction

n-3 DPA (n-3 docosapentaenoic acid)

- Preventive effect of arteriosclerosis (~10 times more effective than EPA)
- Inhibitory effect of angiogenesis



Current n-3 DPA source : **harp seal oil**

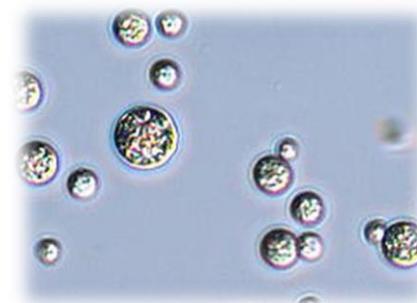
- Low DPA content (< 5%)
- difficulty of stable and large supply



Alternative source such as microorganisms is required.

Labyrinthulomycetes

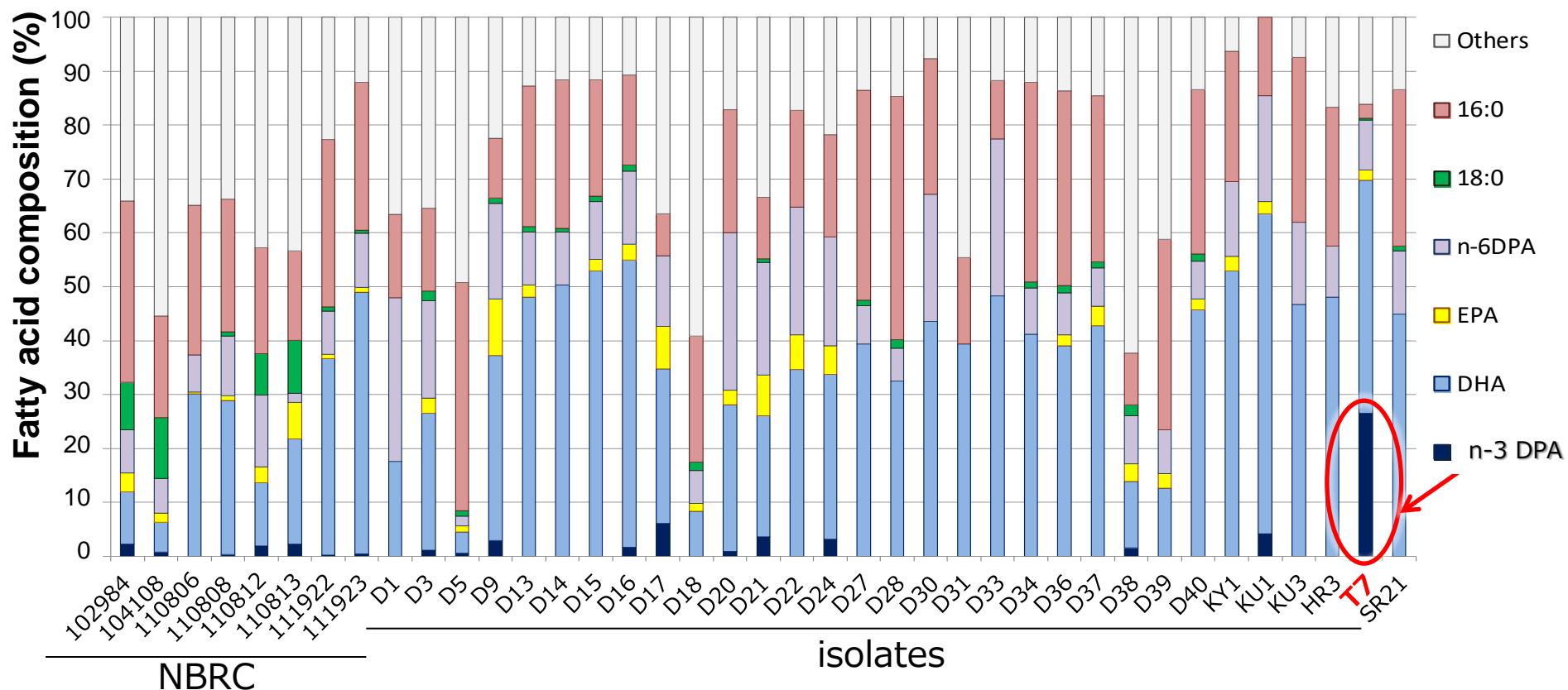
- marine eucaryotic microorganism
- highly accumulate PUFA (including DHA and EPA), astaxanthin, etc.



Isolation of n-3 DPA producing microorganism

— fatty acid compositions of isolates and NBRC strains

31 isolates and 8 NBRC strains were analyzed of fatty acid profiles.



Isolated strain T7 highly accumulated n-3 DPA (26% of total fatty acids).
The strain T7 was identified as *Aurantiochytrium* sp.

1) Polyunsaturated fatty acid production and modification

Various physiological activity

- Cardiovascular fitness
- Cerebral function development
- Anticancer
- Lipid metabolism control
- Immune and inflammation control

Precursor of active metabolites

- **prostaglandin**, leukotriene, thromboxane
- Anti-inflammatory mediators: resolvins etc.

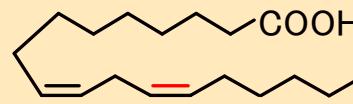
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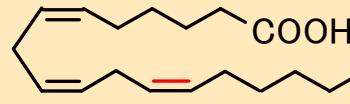


**Microbial oil
as alternative source
Modification of PUFAs
to active metabolites**

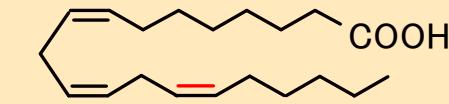
$\omega 6$ (n-6) PUFAs



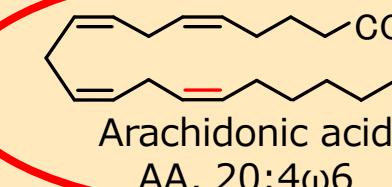
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LA, 18:2 $\omega 6$



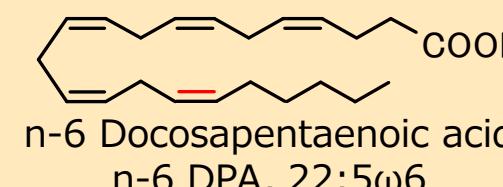
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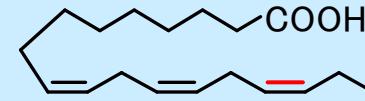


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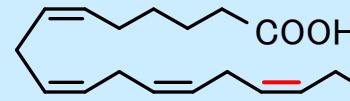


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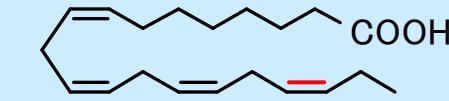
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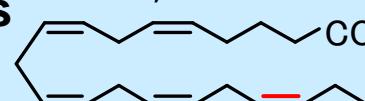
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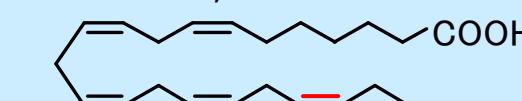
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n-3 Docosapentaenoic acid
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Docosahexaenoic acid
DHA, 22:6 $\omega 3$

Prostaglandins (PG)

Biosynthesis pathway of PGs

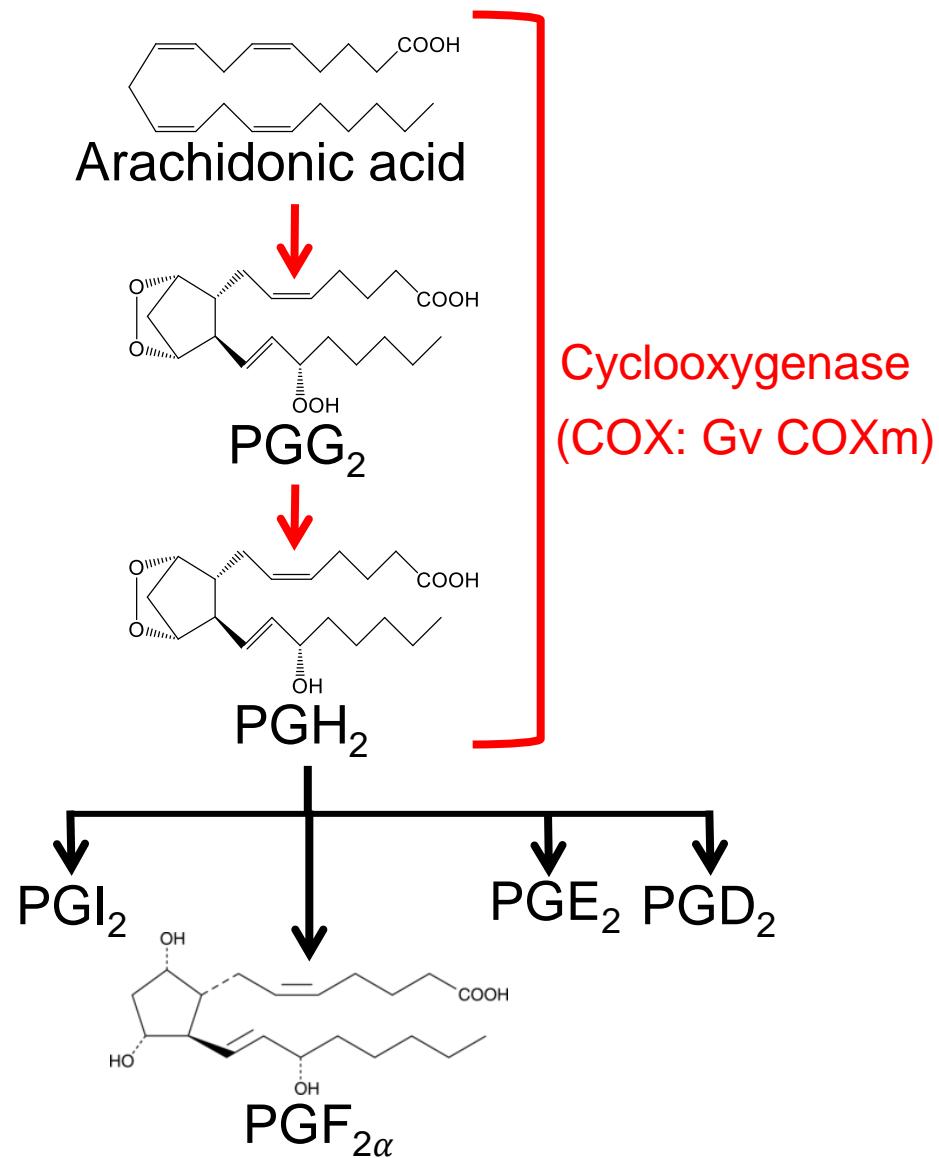
Bioactive substance

PGE₂, PGF_{2α} etc.

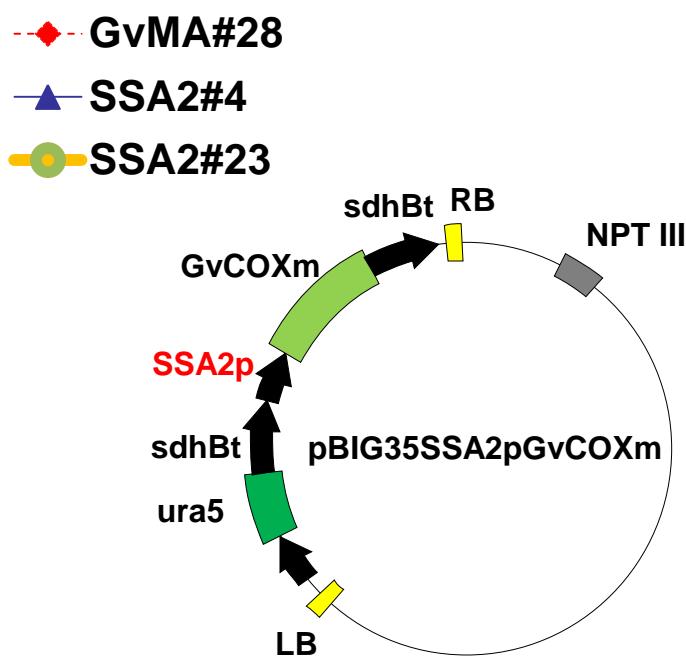
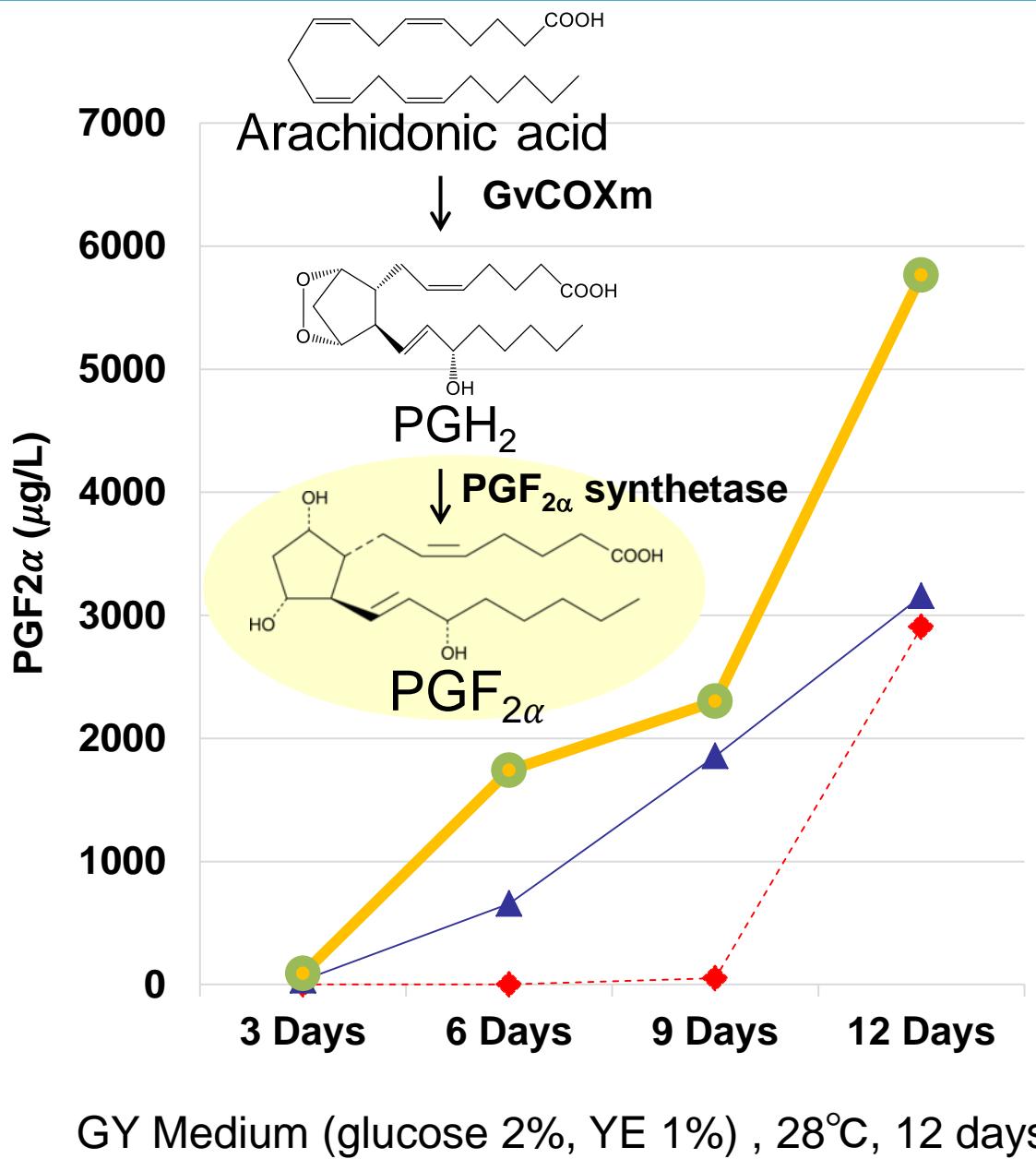
- ✓ Vasodilation
- ✓ Inhibition of platelet aggregation
- ✓ Constriction of smooth muscle



Pharmaceuticals



Time course Analysis of PGF₂ α Production by *Mortierella alpina* 1S-4 transformants



1) Polyunsaturated fatty acid production and modification

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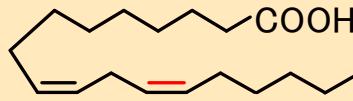
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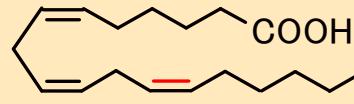


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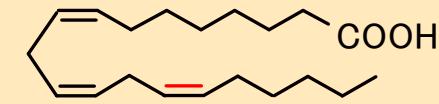
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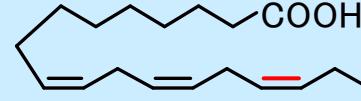


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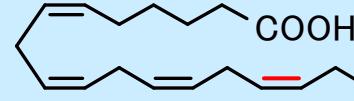


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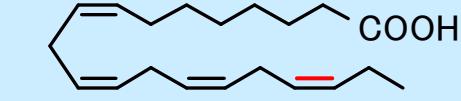
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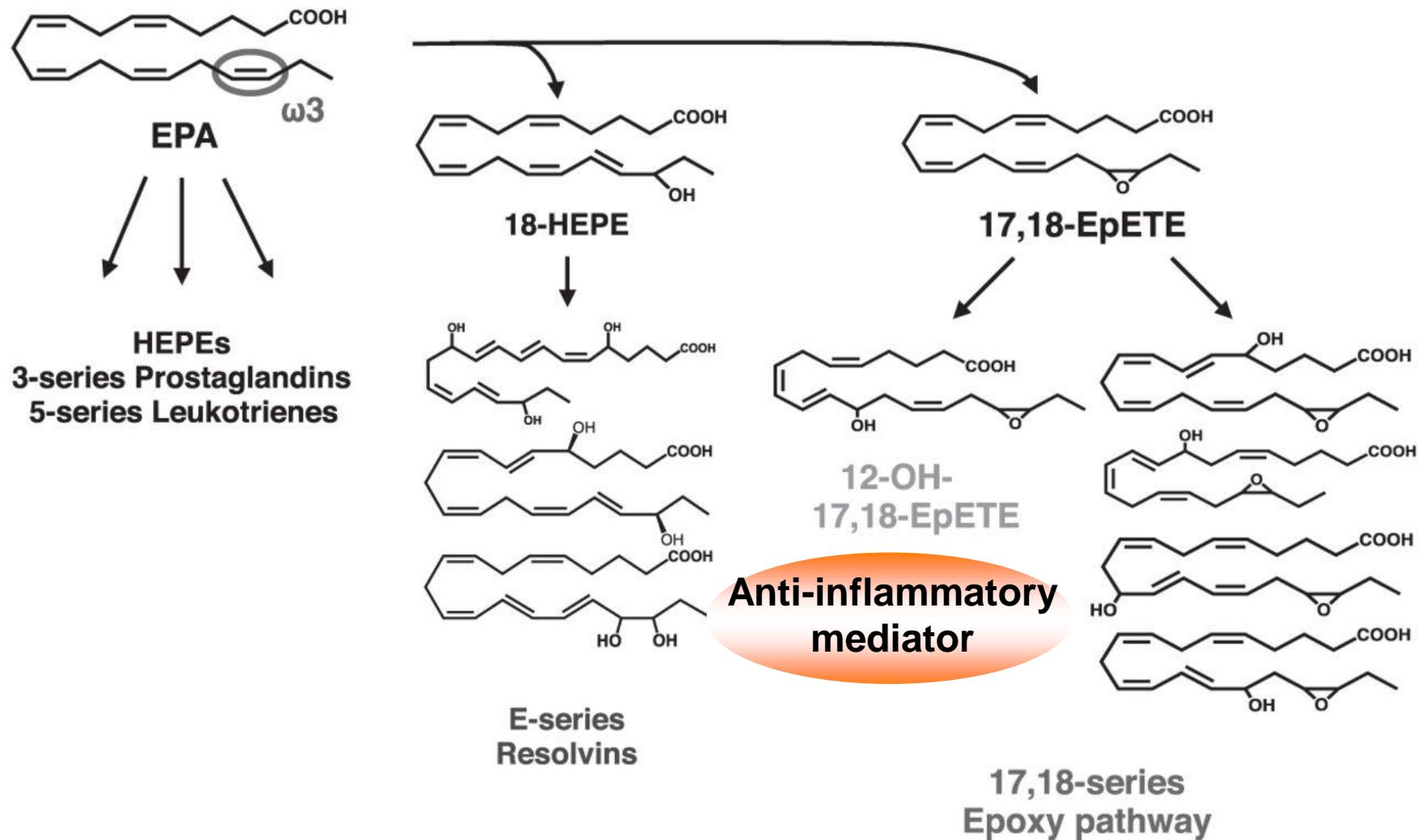


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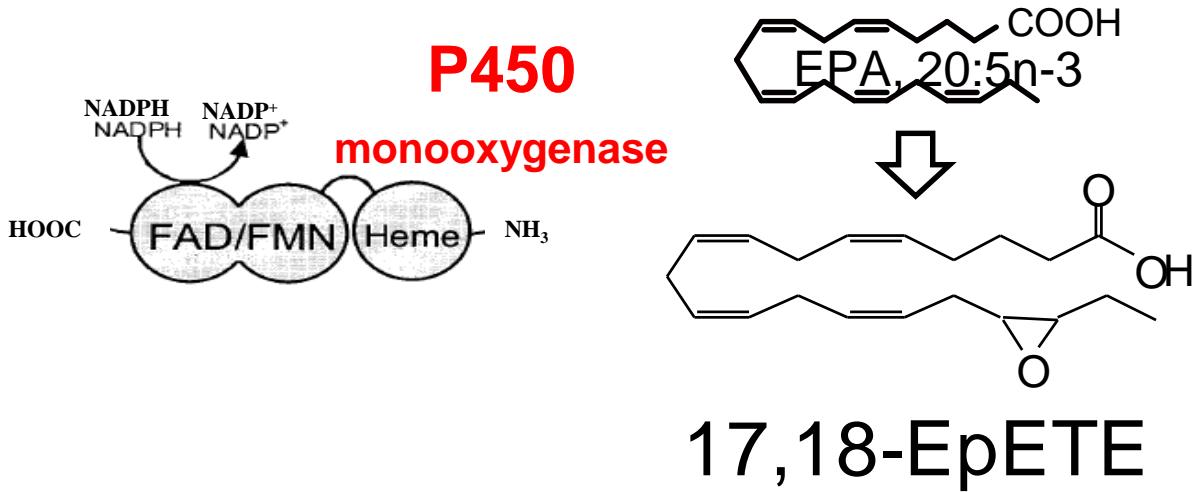


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EPA metabolome in the body



Kubota T et al. FASEB J
2014;28:586-593



Developing new Natto (soy bean fermentation)
containing anti-inflammatory lipids
using *Bacillus* sp. showing P450 monooxygenase activity

Summary

1) Polyunsaturated fatty acid production and modification

Various physiological activity

- Cardiovascular fitness
- Cerebral function development
- Anticancer
- Lipid metabolism control
- Immune and inflammation control

Precursor of active metabolites

- PGF_{2 α} (6 mg/ L) by *M. alpina* transformant
- 17,18-EpETE (0.4 mg/ml) by *Bacillus P450 MO*

Current source...fish oil, plant oil

- unstable supply
- low purity
- contamination of unhealthy compounds



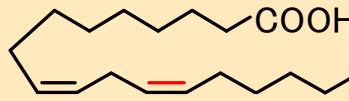
EPA: 52 % 14.4g/L

(*M. alpina* transformant)

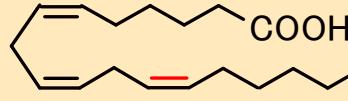
n-3 DPA: 165 mg/L

(*Aurantiochytrium* sp. T7)

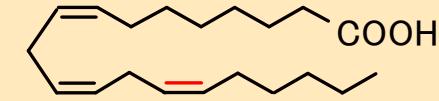
$\omega 6$ (n-6) PUFAs



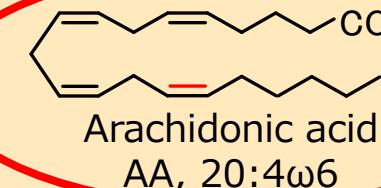
Linoleic acid
LA, 18:2 ω_6



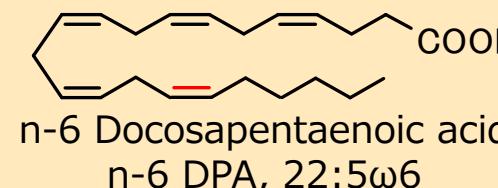
γ -Linolenic acid
GLA, 18:3 ω_6



Dihomo- γ -linolenic acid
DGLA, 20:3 ω_6

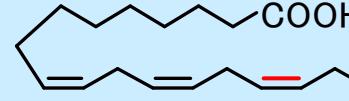


Arachidonic acid
AA, 20:4 ω_6

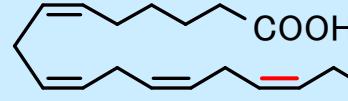


n-6 Docosapentaenoic acid
n-6 DPA, 22:5 ω_6

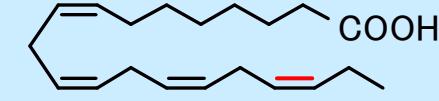
$\omega 3$ (n-3) PUFAs



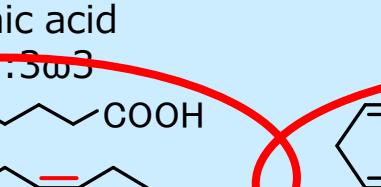
α -Linolenic acid
ALA, 18:3 ω_3



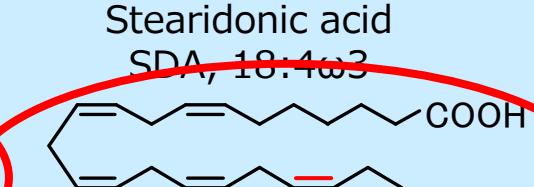
Stearidonic acid
SDA, 18:4 ω_3



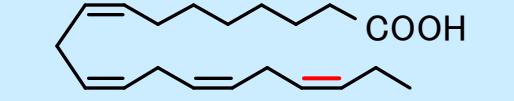
Eicosatetraenoic acid
ETA, 20:4 ω_3



Eicosapentaenoic acid
EPA, 20:5 ω_3

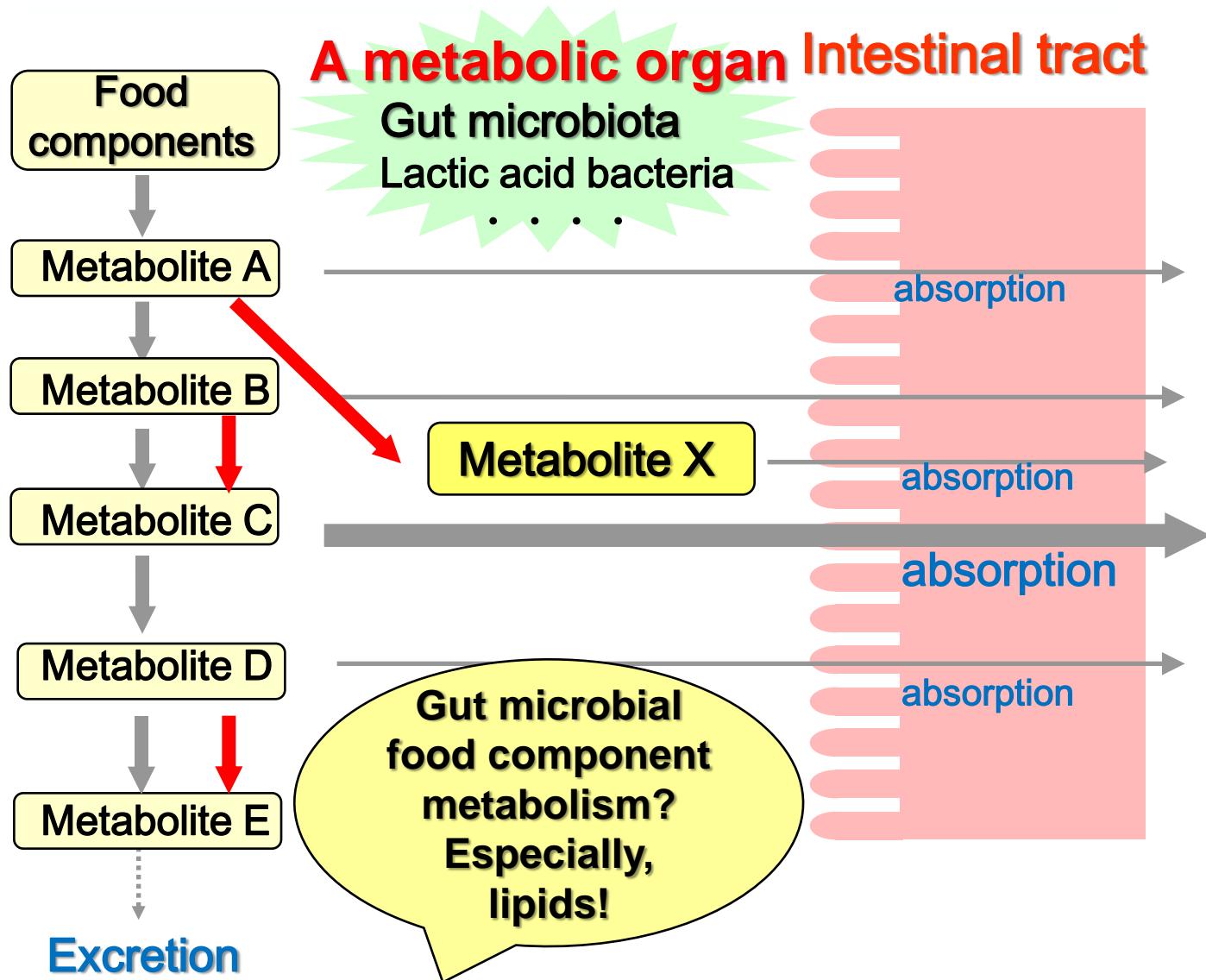


n-3 Docosapentaenoic acid
n-3 DPA, 22:5 ω_3



Docosahexaenoic acid
DHA, 22:6 ω_3

2) Gut microbial fatty acid metabolism

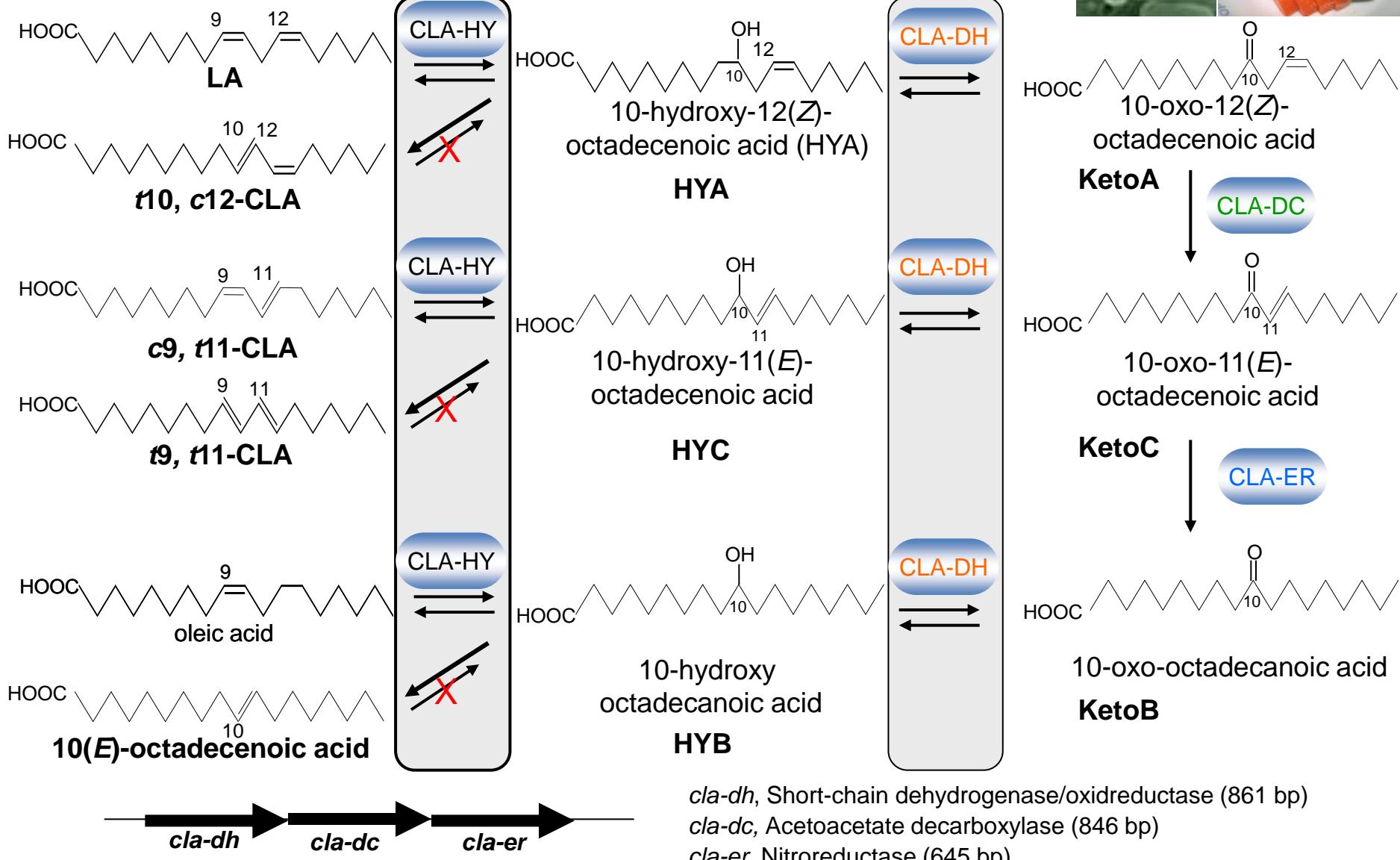
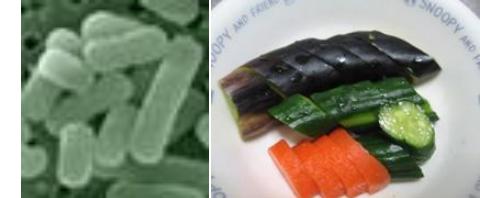


What kind molecular species of fat, especially bioactive PUFAs, are generated by our gut microbes and what kind of function they have in relation with our health?

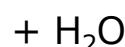
Novel dietary fatty acid metabolism of gut microorganisms producing health-promoting functional lipids

- Gut microbial PUFA saturation metabolism
- Distribution of gut microbial PUFA metabolites in the host
- Physiological activities of gut microbial PUFA metabolites
- Preparation of gut microbial PUFA metabolites

Novel PUFA-saturation metabolism found in *Lactobacillus plantarum*



CLA-HY -Summary-

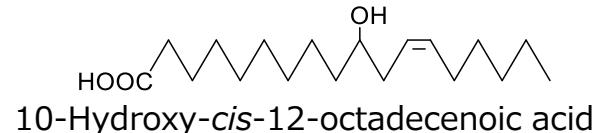


CLA-HY

Hydration



Dehydration



Native M_r
Number of subunit
Cofactor
Activator
Optimal reaction temperature
Optimal reaction pH
Thermal stability
pH stability

(For hydration and dehydration)

52
1
FAD
NADH
40°C
5.5
~28°C
5~7

kDa

His-tagged
CLA-HY →



Marker

(For linoleic acid)

*Sodium succinate buffer pH 5.5, 20 mM;
FAD, 0.1 mM; without NADH; reaction temperature, 37°C

$appK_m$	92	μM
k_{cat}	2.6×10^{-2}	sec ⁻¹
$k_{cat}/appK_m$	2.8×10^{-4}	sec ⁻¹ · μM ⁻¹
Hill coefficient	3.3	
V_{max}	3.1×10^{-2}	μmol · sec ⁻¹ · mg ⁻¹

(For 10-hydroxy-*cis*-12-octadecenoic acid)

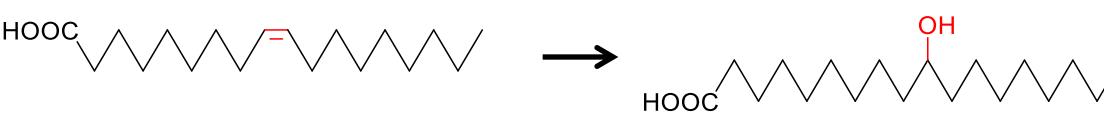
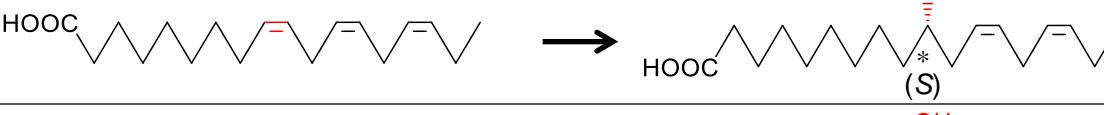
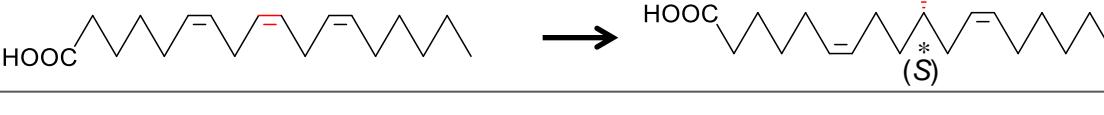
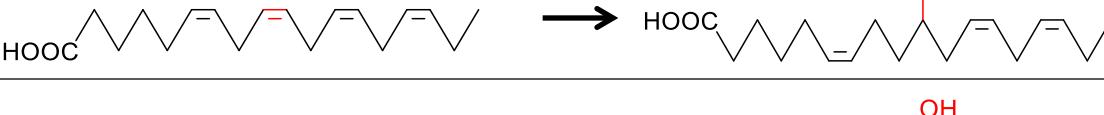
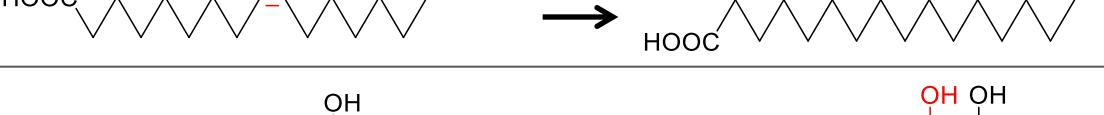
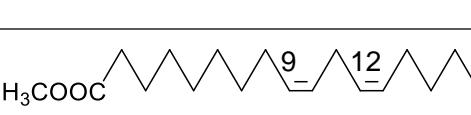
*Sodium succinate buffer pH 5.5, 20 mM;
FAD, 0.1 mM; without NADH; reaction temperature, 37°C

K_m	98	μM
k_{cat}	1.2×10^{-3}	sec ⁻¹
k_{cat}/K_m	1.2×10^{-5}	sec ⁻¹ · μM ⁻¹
V_{max}	1.4×10^{-3}	μmol · sec ⁻¹ · mg ⁻¹

CLA-HY -Substrate specificity-

Substrates (for hydration)

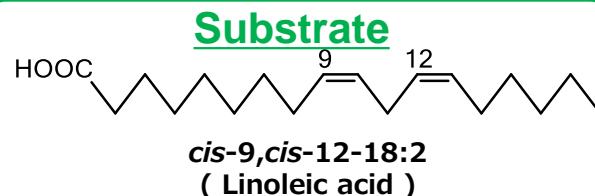
Relative activity (%)

Substrates (for hydration)		Relative activity (%)
Linoleic acid (18:2)		<u>100</u>
Oleic acid (18:1)		<u>335</u>
α-Linolenic acid (18:3)		<u>29</u>
γ-Linolenic acid (18:3)		<u>43</u>
Stearidonic acid (18:4)		<u>43</u>
Palmitoleic acid (16:1)		<u>44</u>
Ricinoleic acid (12-OH 18:1)		<u>0.5</u>
cis-Vaccenic acid (cis-11-Octadecenoic acid) (18:1)		—
Elaidic acid (trans-9-Octadecenoic acid) (18:1)		—
Methyl linoleate (18:1 methyl ester)		—

Linoleic acid metabolism by *Pediococcus* sp. AKU 1080

$\Delta 9$ hydration pathway
(C10-OH FA)

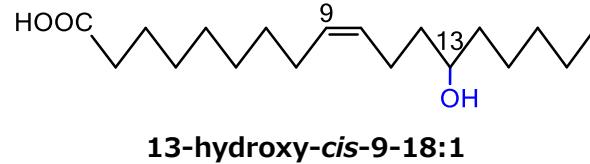
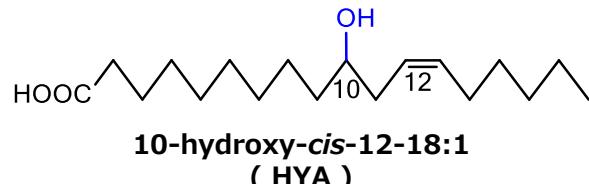
$\Delta 12$ hydration Pathway
(C13-OH FA)



CLA-HY

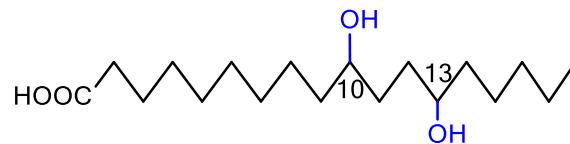
Hydroxy fatty acids

FA-HY

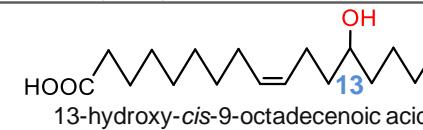
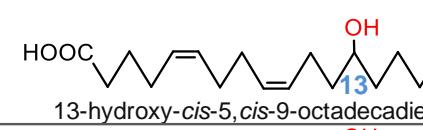
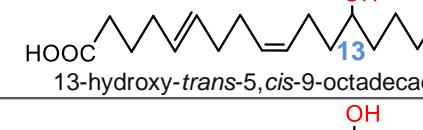
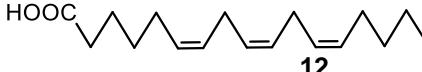
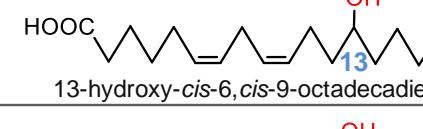
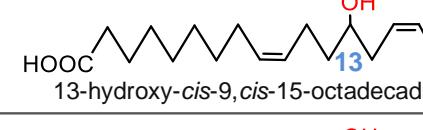
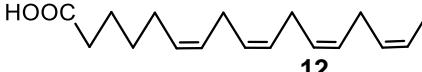
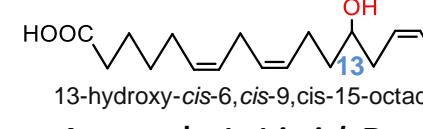


FA-HY

CLA-HY

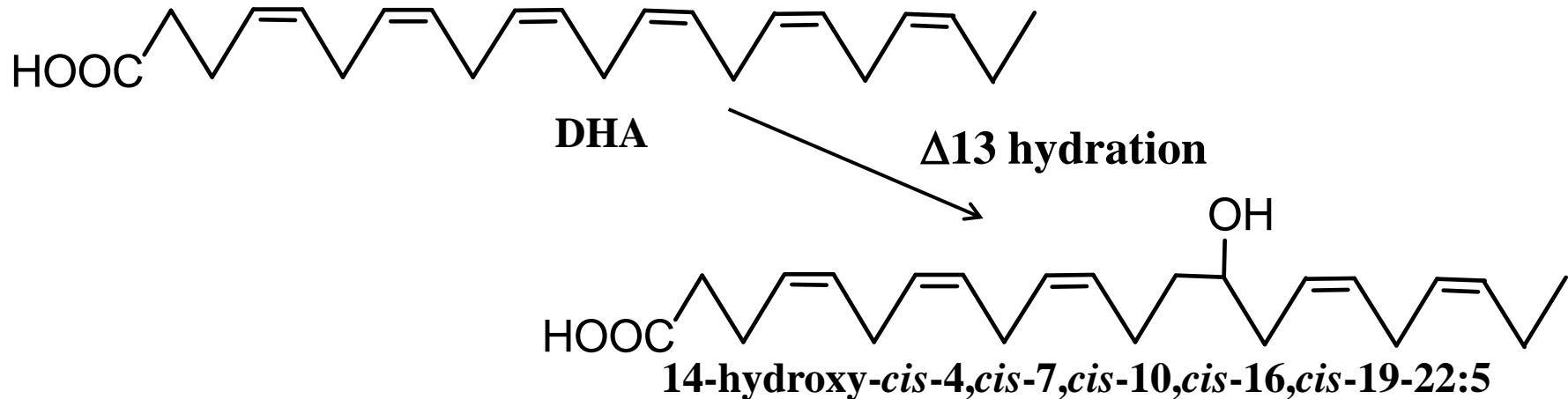


Hydration reactions catalyzed by *L. acidophilus* hydratase (FA-HY1)

	Substrate (10 mM)	Product	Yield (%) in 16 h reaction
Palmitoleic acid C16:1 Δ^{9cis}			2
Oleic acid C18:1 Δ^{9cis}			1
cis-Vaccenic acid C18:1 Δ^{11cis}			59
Linoleic acid C18:2 $\Delta^{9cis}, \Delta^{12cis}$			48
C18:2 $\Delta^{9trans}, \Delta^{12trans}$		Not hydrated	
Pinolenic acid C18:3 $\Delta^{5cis}, \Delta^{9cis}, \Delta^{12cis}$			57
Columbinic acid C18:3 $\Delta^{5trans}, \Delta^{9cis}, \Delta^{12cis}$			46
γ -Linolenic acid C18:3 $\Delta^{6cis}, \Delta^{9cis}, \Delta^{12cis}$			57
α -Linolenic acid C18:3 $\Delta^{9cis}, \Delta^{12cis}, \Delta^{15cis}$			54
Stearidonic acid C18:4 $\Delta^{6cis}, \Delta^{9cis}, \Delta^{12cis}, \Delta^{15cis}$			12

Substrate specificity of fatty acid hydratase (FA-HY1) from *Lactobacillus acidophilus* NTV001

Substrate	Product
20:2 $\Delta^{11}cis, \Delta^{14}cis$	15-hydroxy- <i>cis</i> -11-20:1
20:3 $\Delta^{5}cis, \Delta^{8}cis, \Delta^{11}cis$	12-hydroxy- <i>cis</i> -5, <i>cis</i> -8-20:2
20:3 $\Delta^{5}cis, \Delta^{11}cis, \Delta^{14}cis$	15-hydroxy- <i>cis</i> -5, <i>cis</i> -11-20:2
20:3 $\Delta^{8}cis, \Delta^{11}cis, \Delta^{14}cis$ (DGLA)	15-hydroxy- <i>cis</i> -8, <i>cis</i> -11-20:2 12-hydroxy- <i>cis</i> -8, <i>cis</i> -14-20:2
20:3 $\Delta^{11}cis, \Delta^{14}cis, \Delta^{17}cis$	12-hydroxy- <i>cis</i> -14, <i>cis</i> -17-20:2 15-hydroxy- <i>cis</i> -11, <i>cis</i> -17-20:2
20:4 $\Delta^{5}cis, \Delta^{8}cis, \Delta^{11}cis, \Delta^{14}cis$ (AA)	15-hydroxy- <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11-20:3
20:4 $\Delta^{8}cis, \Delta^{11}cis, \Delta^{14}cis, \Delta^{17}cis$	12-hydroxy- <i>cis</i> -8, <i>cis</i> -14, <i>cis</i> -17-20:3
EPA	—
DHA	14-hydroxy-<i>cis</i>-4,<i>cis</i>-7,<i>cis</i>-10,<i>cis</i>-16,<i>cis</i>-19-22:5

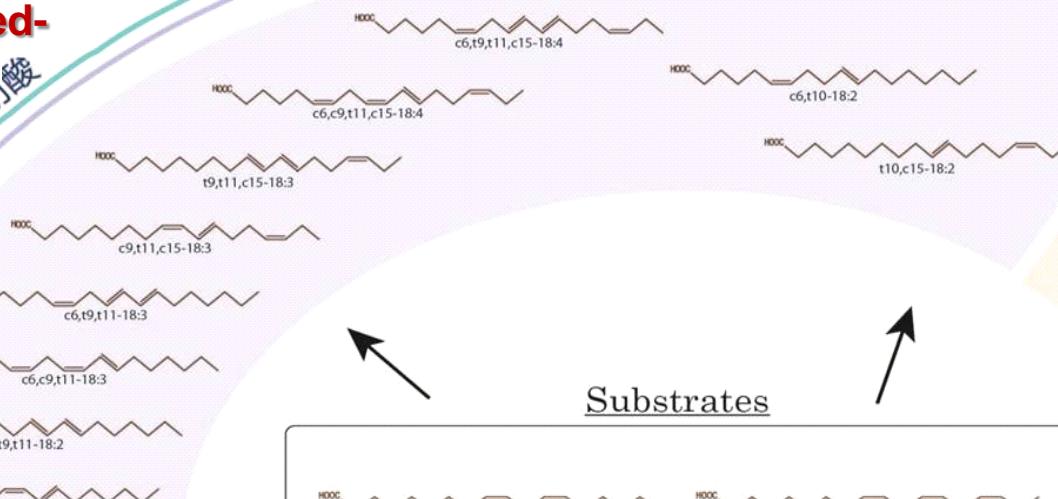


非メチレン型脂肪酸

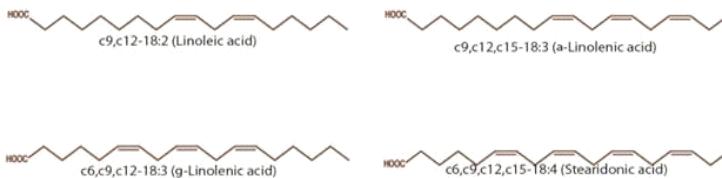
Non-methylene interrupted-

Conjugated-

共役型脂肪酸

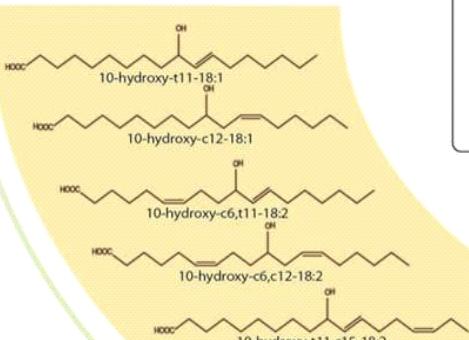


Substrates



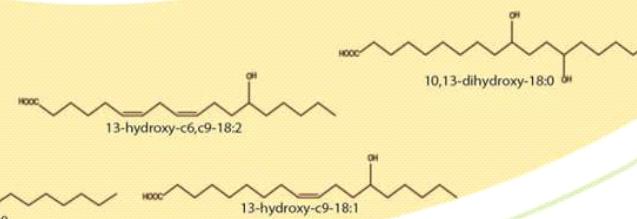
Oxo-

オキソ脂肪酸

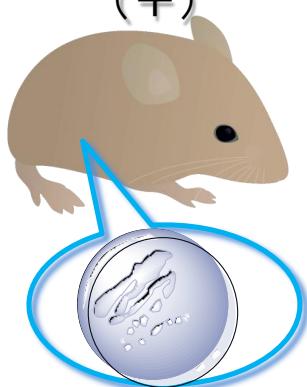


Hydroxy-

水酸化脂肪酸



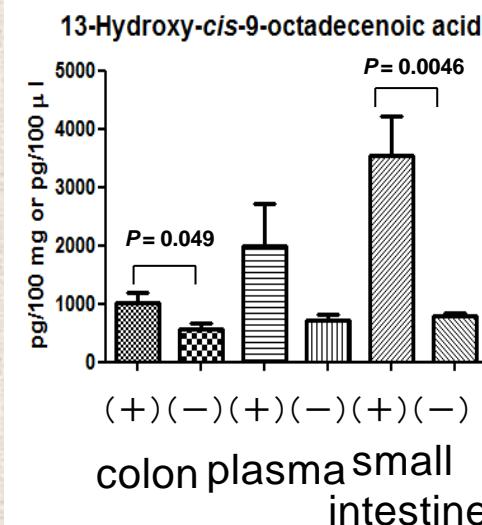
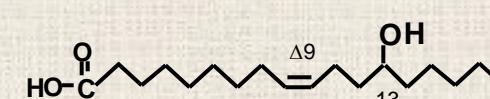
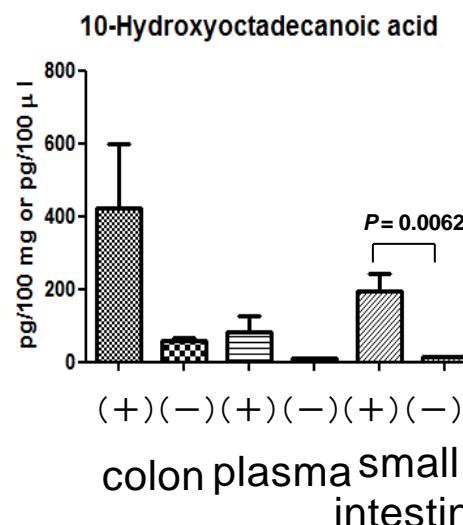
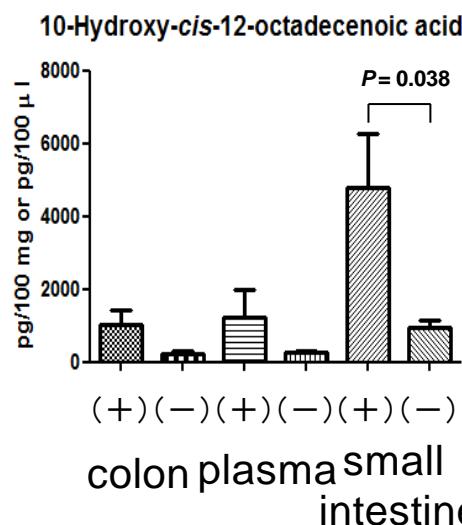
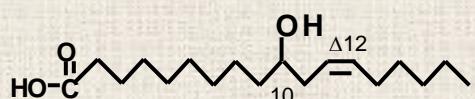
With gut microbes
(specific pathogen-free)
(+)



Without gut microbes
(germ-free)
(-)



VS



The concentrations are 0.01 to 0.1 μM

Enhancing gut hormone secretion & anti-diabetic activity

- Goto J. et al, *Biochem. Biophys. Res. Commun.*, 459, 597-603 (2015)
Kim M. et al, *FASEB J.*, 31, 5036-5048 (2017)
Yonejima Y., et al, *Prog. Med.*, 37, 1105-1111 (2017)
Miyamoto J., et al, *Nat. Commun.* 10, 4007 (2019)

Hypolipidemic effect

- Nanthirudjanar T., et al, *Lipids*, 50, 1093-1102 (2015)

Anti-inflammatory and immune controlling effects

- Bergamo P., et al, *J. Funct. Foods*, 11, 192-202 (2014)
Ohue-Kitano R., et al, *FASEB J.*, 32, 304-318 (2018)

Intestinal and gingival epithelial barrier protection

- Miyamoto J. et al, *J. Biol. Chem.*, 290, 2902-2918 (2015)
Yamada M. et al., *Sci. Rep.*, 8, 9008 (2018)

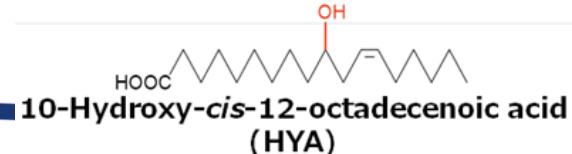
Anti-oxidative effects

- Furumoto H., et al, *Toxicol. Appl. Pharmacol.*, 296, 1-9 (2016)

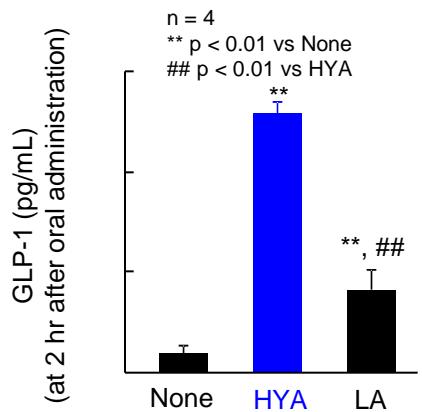
Anti *Helicobacter pylori* activity

- Matsui H., et al, *Helicobacter*, 22, e12430 (2017)

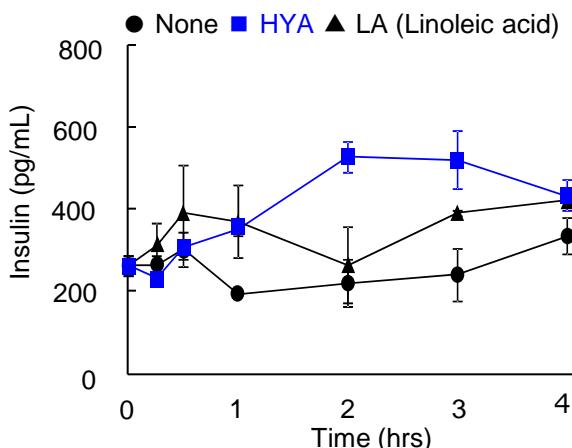
HYA induced gut hormone secretion



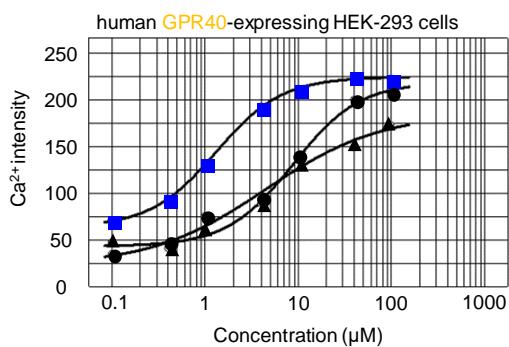
Oral administration of HYA (1000 mg/kg) induces insulin secretion by increasing GLP-1 level



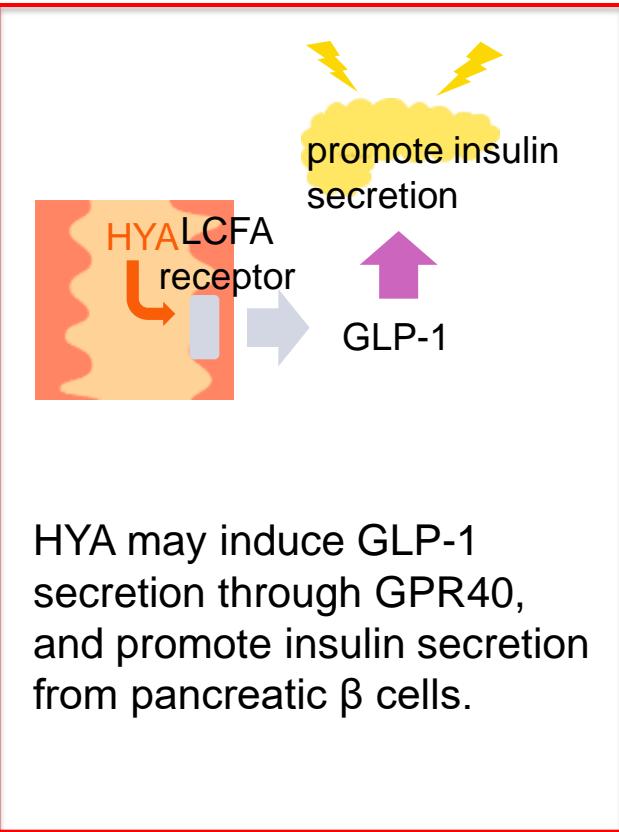
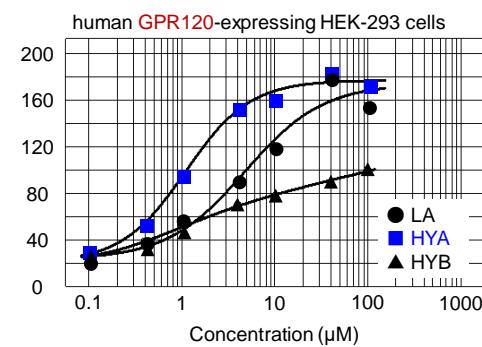
Blood GLP-1 concentration



Blood Insulin concentration



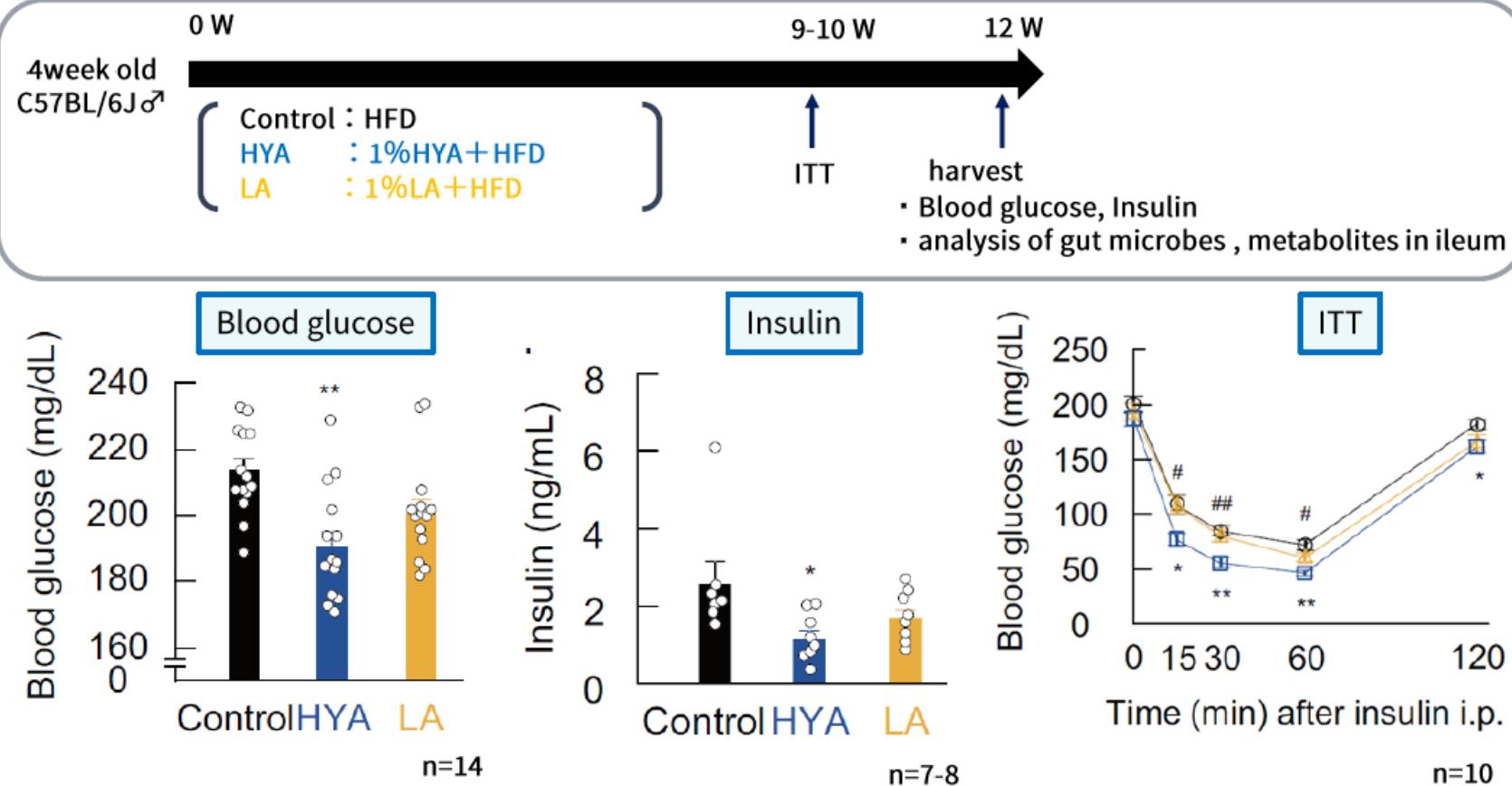
Affinity of GPR40 and GPR120



HYA may induce GLP-1 secretion through GPR40, and promote insulin secretion from pancreatic β cells.

Miyamoto J. The 2016 annual conference of the Japan Society for Bioscience, Biotechnology and Agrochemistry.

HYA improves insulin resistance



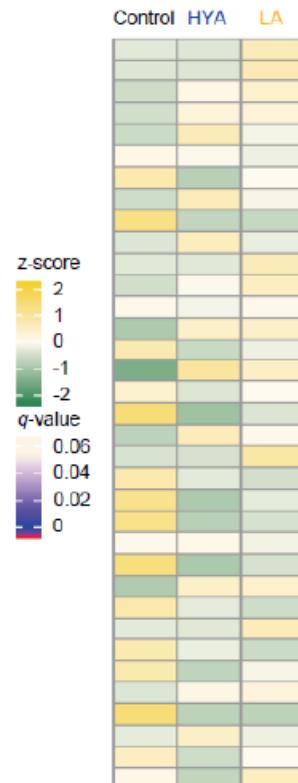
HYA improves HFD-induced insulin resistance by promoting GPR40 and 120-mediated GLP-1 secretion.

HYA alters gut microbiome and their metabolites



12 weeks administration
Control : HFD
HYA : 1%HYA+HFD
LA : 1%LA+HFD

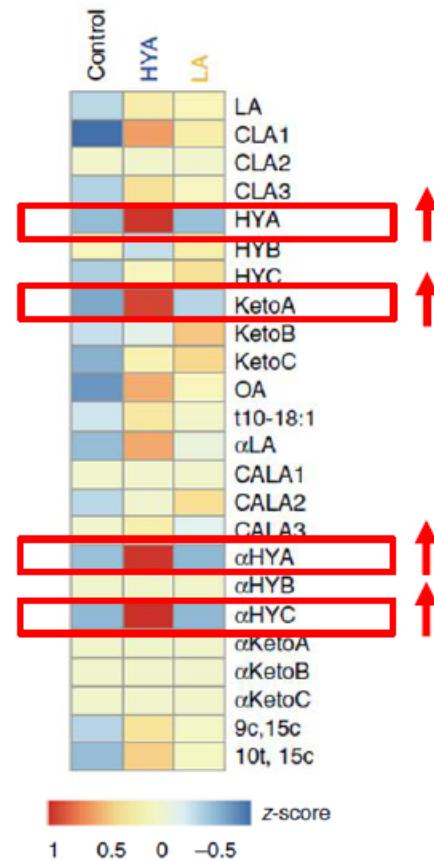
Gut microbial composition



Control vs HYA
Control vs LA
HYA vs LA

Bifidobacteriaceae
Microbacteriaceae
Corymbacteriaceae
Bacteroidaceae
Bacteroidales S24-7 group
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Deferribacteraceae
Bacillaceae
Bacillales_Family XI
Staphylococcaceae
Aerococcaceae
Carnobacteriaceae
Enterococcaceae
Lactobacillaceae
Suepratoccaceae
Christensenellaceae
Clostridiaceae I
Clostridiales vadimBB60 group
Clostridiales_Family XIII
Lachnospiraceae
Peptococcaceae
Peptostreptococcaceae
Ruminococcaceae
Erysipelotrichaceae
Acidaminococcaceae
Veillonellaceae
Phyllobacteriaceae
Alcaligenaceae
Burkholderiaceae
Desulfovibrionaceae
Holobiacteraceae
Enterobacteriaceae
Verrucomicrobiaceae

Gut microbial PUFA metabolites



Miyamoto, J. et al, *Nat. Commun.* (2019)

HYA increase the abundance of the *Lactobacillaceae* family and specific metabolites in ileum

Dietary PUFAs

$\omega 3$ fatty acid

α -Linolenic acid

DHA,EPA

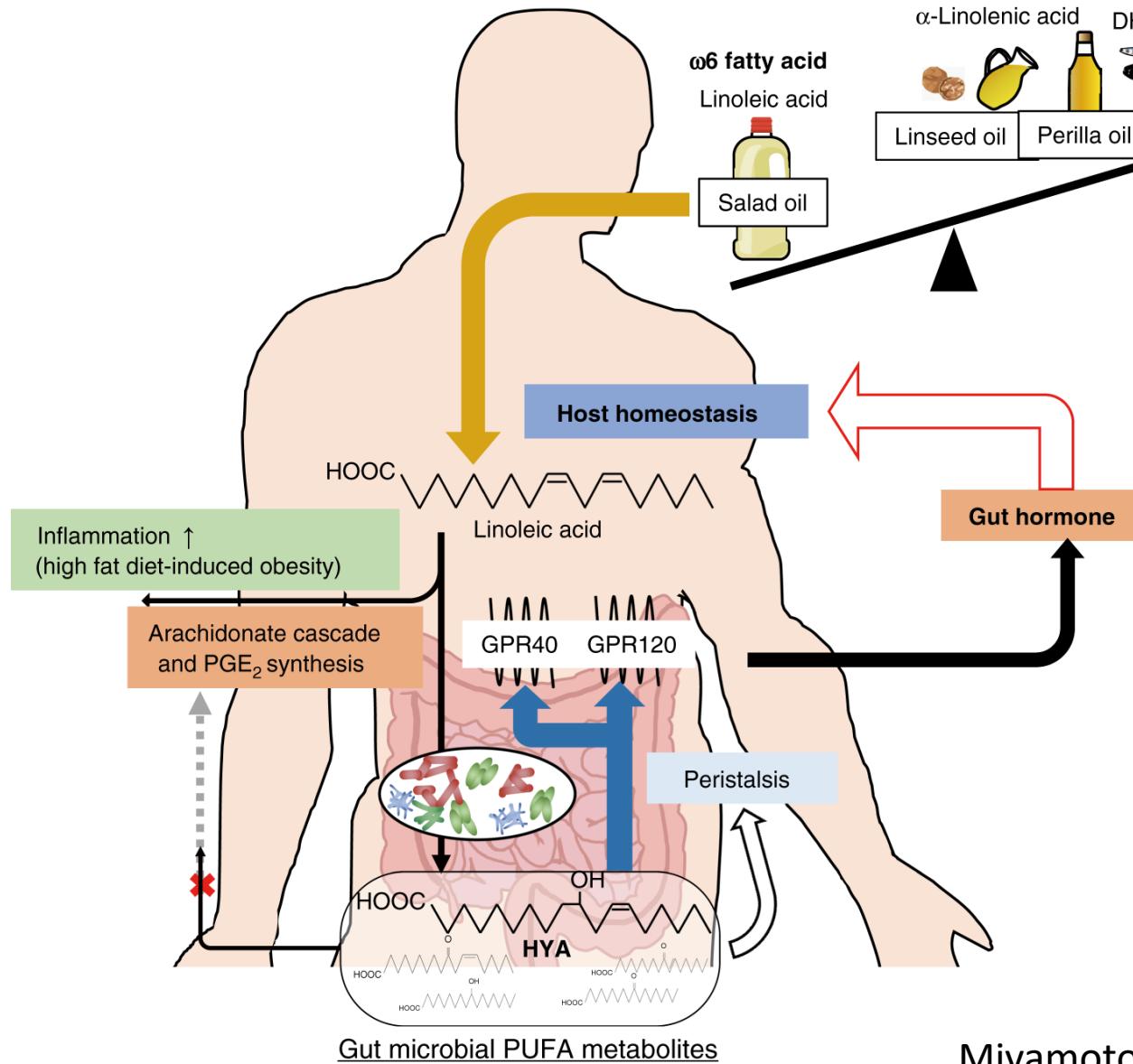
Linseed oil

Perilla oil

$\omega 6$ fatty acid

Linoleic acid

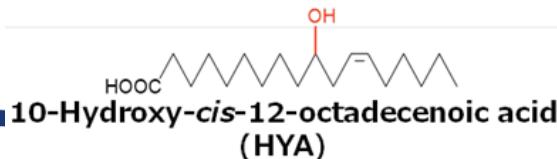
Salad oil



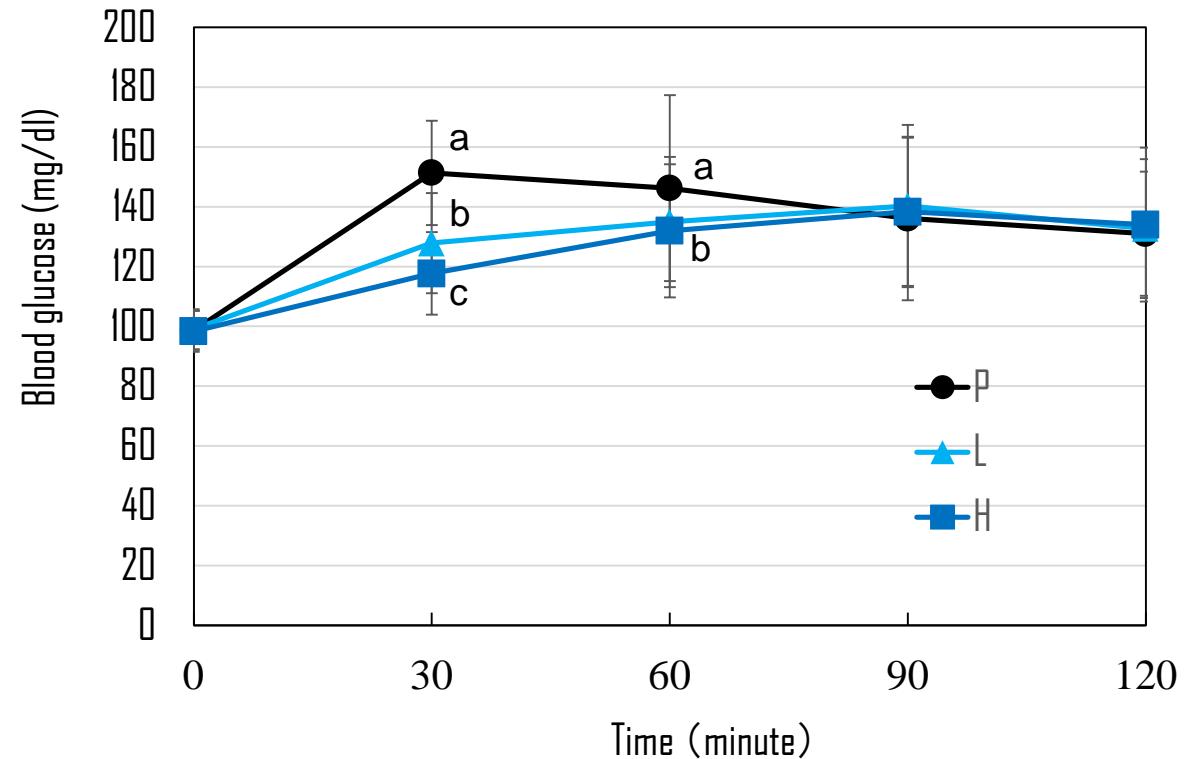
Miyamoto J., et al,
NATURE COMMUNICATIONS
10:4007, 2019

Gut microbiota confers host resistance to obesity by metabolizing dietary polyunsaturated fatty acids

Clinical trial results



Changes in blood glucose after ingestion

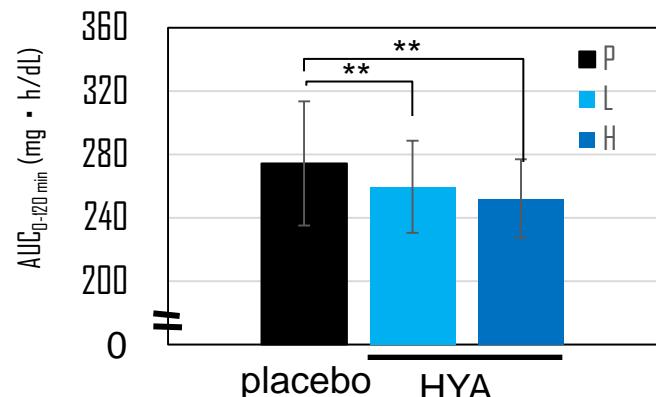


P: ingesting a placebo

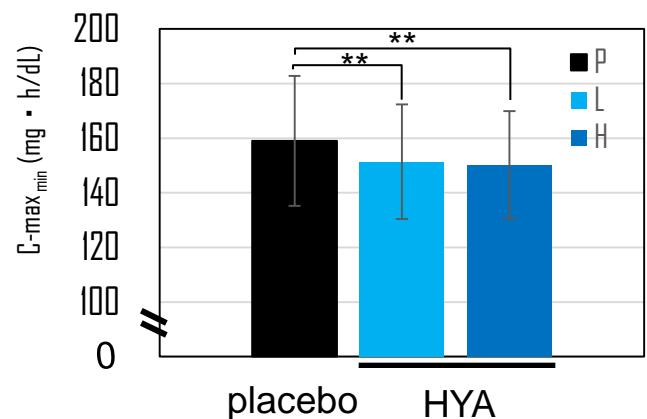
L : ingesting low dose HYA (1000mg)

H : ingesting high dose HYA (2000mg)

AUC_{0-120 min}



C-max min



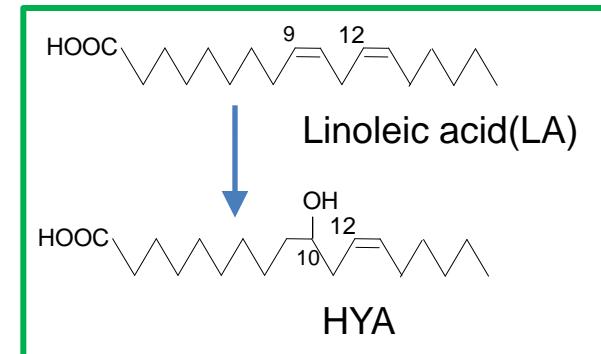
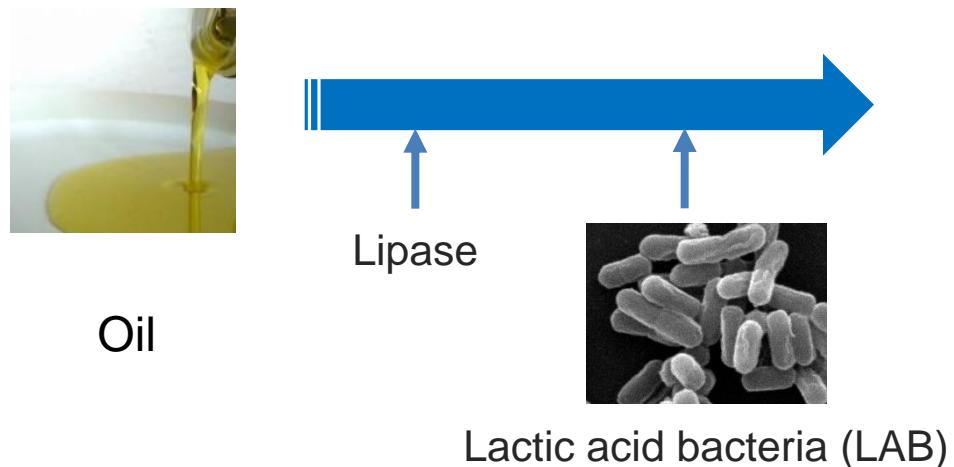
Hydroxy fatty acid production using CLA-HY expressed in *E. coli*

Substrates (280 g/L)

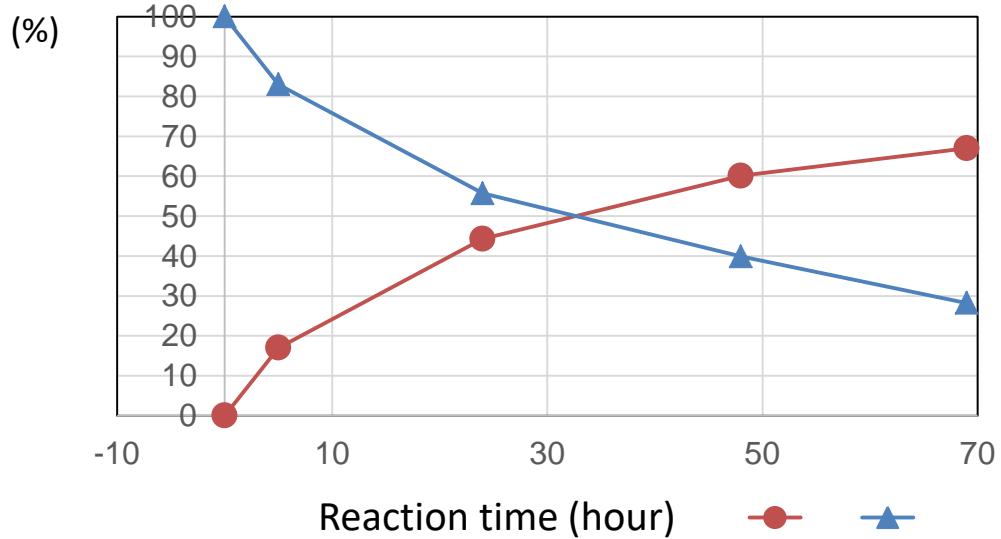
Yield [%]

Linoleic acid (18:2)			98% (292 g/L)
Oleic acid (18:1)			96% (286 g/L)
α-Linolenic acid (18:3)			96% (286 g/L)
γ-Linolenic acid (18:3)			95% (283 g/L)
Stearidonic acid (18:4)			96% (286 g/L)
Palmitoleic acid (16:1)			98% (294 g/L)
Ricinoleic acid (12-OH 18:1)			99% (294 g/L)

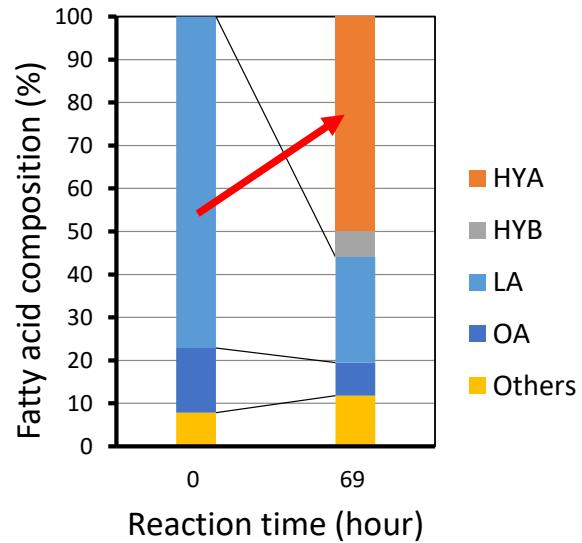
HYA production from edible plant oils using lactic acid bacteria



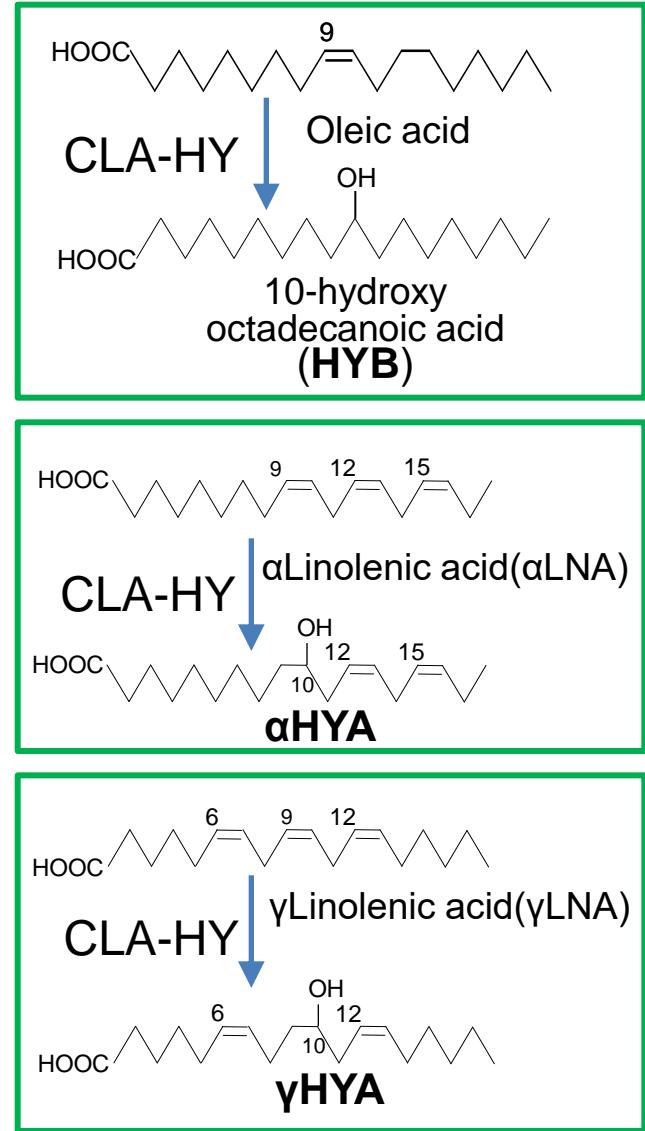
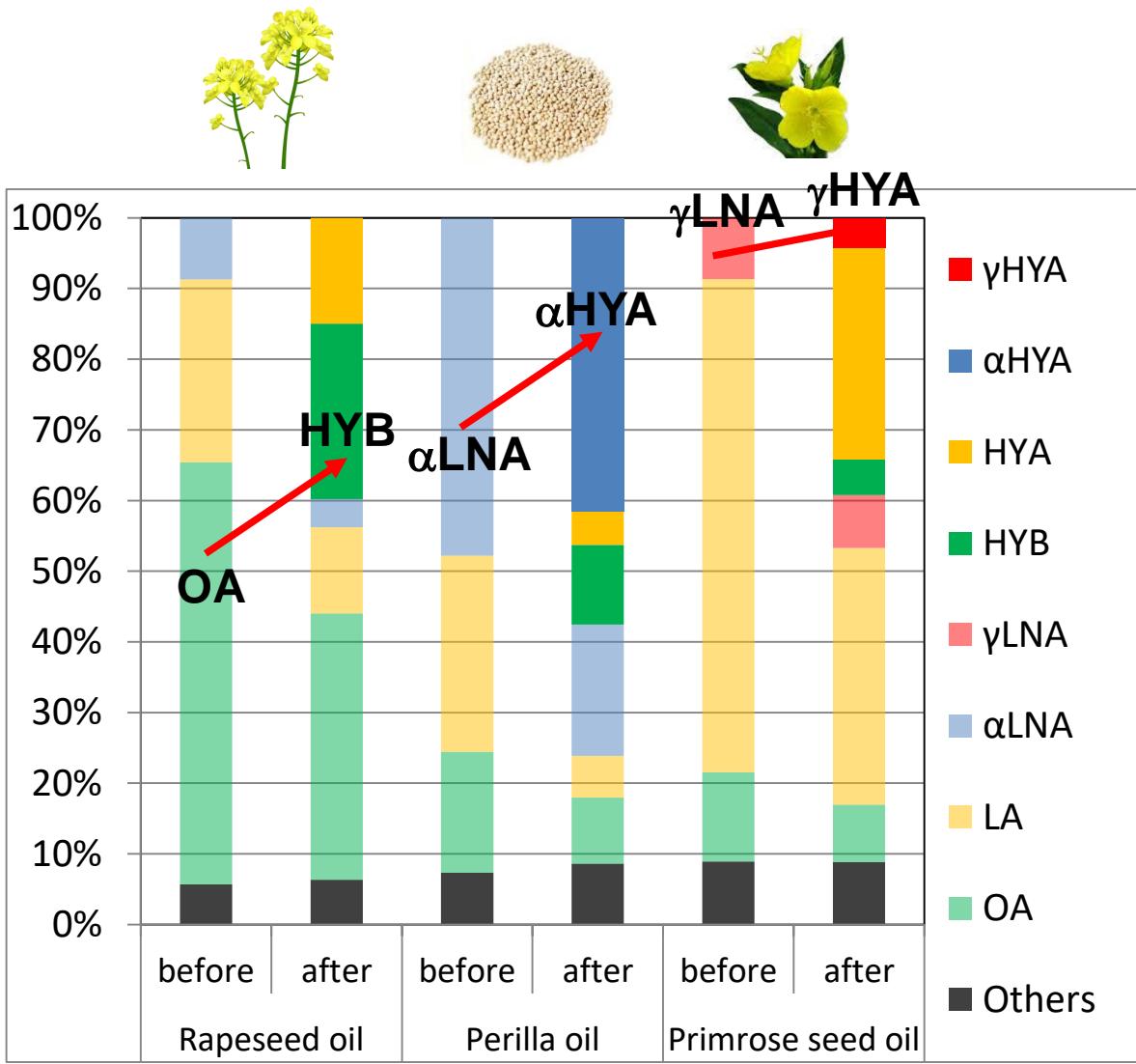
Time course of HYA production



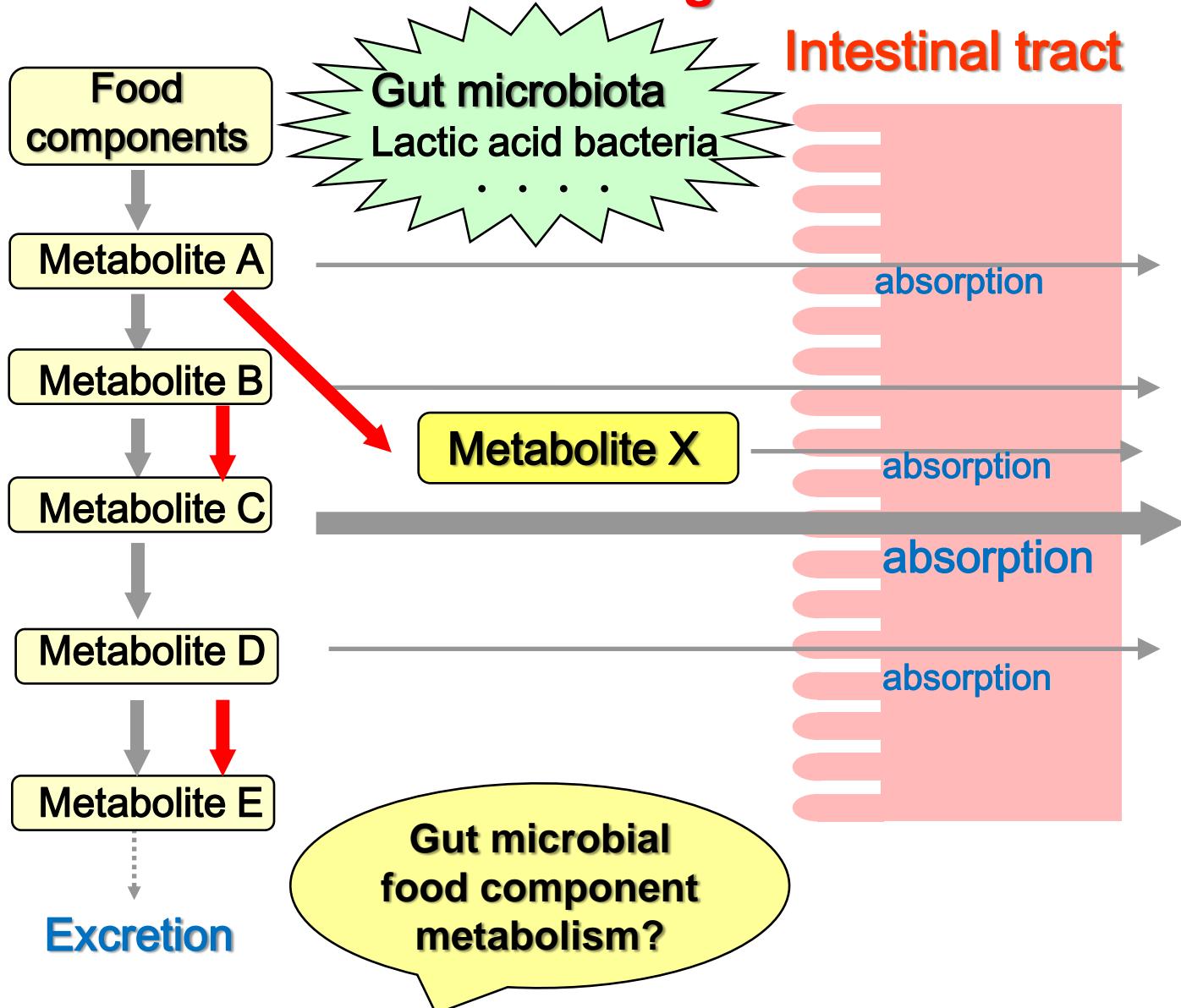
Fatty acid composition after reaction



HYA production from edible plant oils using lactic acid bacteria



A metabolic organ

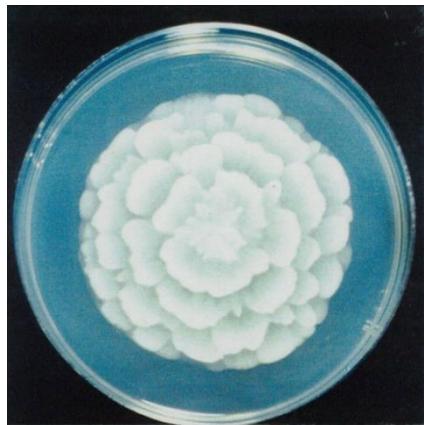


for Human Health and Good Life

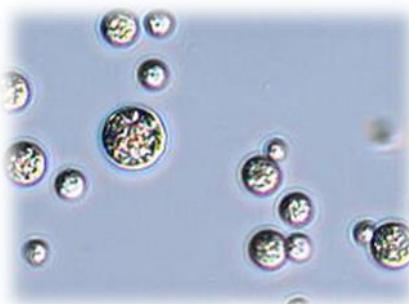
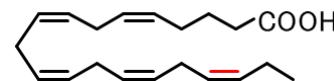
Summary

Functional lipid production by microbial metabolisms and enzymes: beyond common polyunsaturated fatty acids

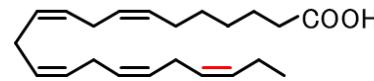
Fermentative production of PUFAs



EPA: 52 % 14.4g/L
M. alpina transformant
expressing
 $\Delta 12, \Delta 17$ desaturases



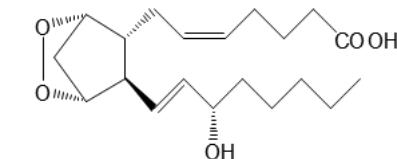
n-3 DPA: 165 mg/L
Aurantiochytrium sp. T7



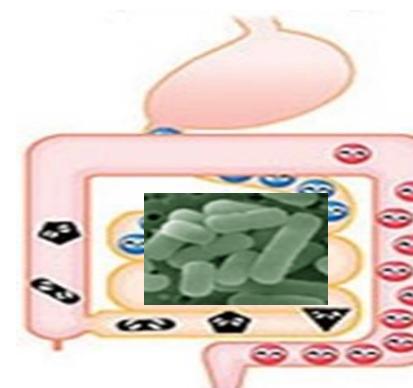
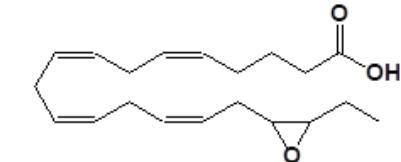
Biotransformation of PUFAs



PGF_{2 α} : 6 mg/L
M. alpina transformant
expressing
COX from alga



17,18-EpETe:
0.38 mg /mL
P450 MO from
Bacillus species



HYA: 292 g /L
Other hydroxy FAs,
Oxo-FAs, enone FAs
Lactobacillus species



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Thank you for your kind attention !

