Supplementary Method and Results

Plasmid construction and transformation in *B. subtilis*

To clone the ADH4 gene, the gene was PCR amplified with primers having Kpn I and Hind III site at 5' and 3' end, respectively, with denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at 55 °C for 15 sec, extension at 72 °C for 30 sec and a final elongation for 10 min.

The purified product and plasmid pBE-S (1 µg) were digested with selected restriction enzymes, and ligation was performed per standard procedure. The ligated product was transformed into competent cells of *E.coli* DH-5α, and transformants were confirmed by colony PCR and further by sequencing. Thereafter, a plasmid was isolated from a single colony of pBE-S:ADH4 transformant and it was transformed to competent *B. subtilis* RIK 1285 by electroporation (1.5KV,100 Ω, 25µF, time constant 1.5 milli second) and plated on a LB-agar plate supplemented with kanamycin (10 µg/ml). The obtained transformants with pBE-S:ADH4 were screened by colony PCR and sequencing. The selected transformant RIK 1285:ADH4 (F1) was tested for its *in-vitro* ethanol (0.5%) removal efficiency.

Protein expression and signal peptide library

The obtained pellet was washed with PBS buffer twice to remove any presence of media. To extract the pellet, pellet was suspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0; 1mM EDTA, pH 8.0; 100 mM NaCl, glycerol 10%, 40 mM DTT, 1 mM PMSF, 10 mg/ml lysozyme) for 15 min. The pellet was disrupted with an ultrasonic homogenizer using the cycle, work 8 sec, stop 10 sec and centrifuged at 12,000 rpm for 15 min at 4 °C to obtain the supernatant. Proteins were quantified by the standard Bradford method, and an equal amount of protein was loaded on a 12% SDS-PAGE. The resulting gels were transferred to a nitrocellulose membrane and blocked for 1 h at room temperature in a 5% (W/V) skim-milk (Difco Laboratories) in TBS buffer (20 mM Tris, 100 mM NaCl, 0.1% Tween-20). After blocking, the membrane was incubated overnight with gentle agitation at 4 °C with the Primary antibody (Rabbit,1:2000). Following antibody treatment, the membrane was washed 3 times for 5 min each with 25 ml TBS buffer and incubated with the HRP-conjugated secondary antibody (anti-rabbit, 1:5000) for 30 min at room temperature. After incubation, the membrane was washed 3 times with TBS buffer and further subjected to enhanced chemiluminescence (Biorad, USA). A small fragment of 710bp constructed strain was used as a control for protein expression.

To construct the signal-peptide library, plasmid pBE:S:ADH4 was digested with restriction enzymes MluI and Eco52 I, and randomly ligated with SP DNA mixture (Takara Biosciences).

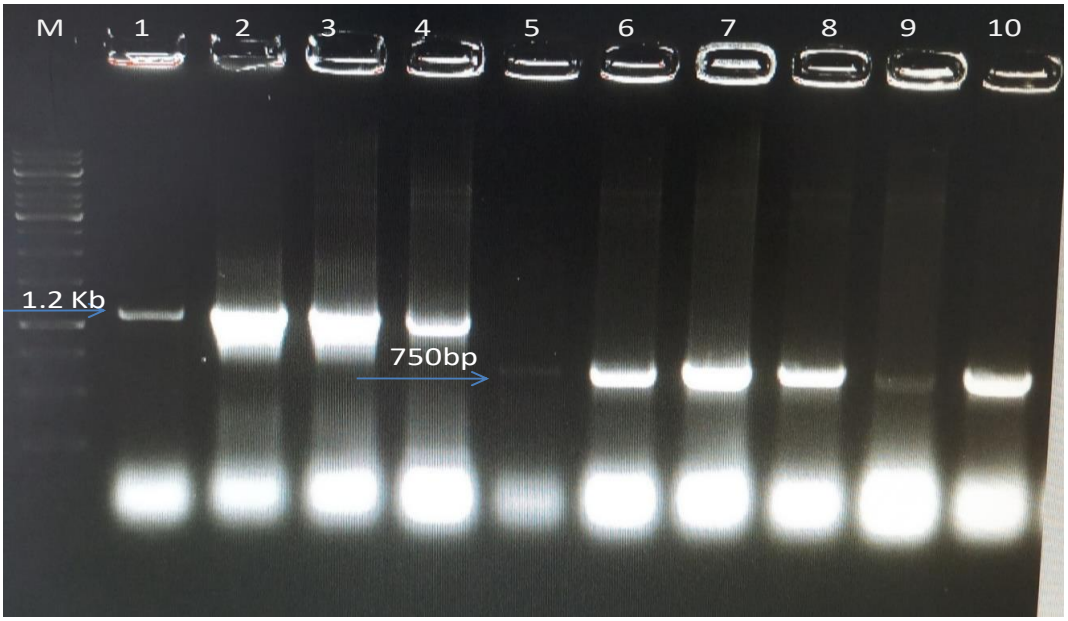
E. coli HST08 (Stellar) competent cells were used to create the pBE-S:ADH4 random signal peptide plasmid library. Finally, the plating was done on an LB-agar plate containing ampicillin (100 µg/ml). Approximately 2,000 antibiotic-resistant transformants were pooled and suspended in LB- broth to purify the pBE-S:ADH4 random signal peptide plasmid library. The plasmid library was transformed into *B. subtilis* RIK 1285 by electroporation and plated on LB-agar medium added with kanamycin (10 µg/ml). Approximately 100 clones were propagated and screened for the *in-vitro* ethanol removal assay, and selected colonies were tested for alcohol dehydrogenase (ADH) activity.

In-vitro ethanol removal assay

The column used was an HP-FFAP (Agilent Technologies, USA) equipped with a capillary column (30 m × 0.53 mm × 1.0 µm, Agilent Technologies, USA) and a FID (flame ionization detector). The injector and detector temperatures were set at 200 °C and 240 °C, respectively. The initial GC oven temperature of 120 °C was held for 1 min and then ramped at 10 °C/min to a final temperature of 240 °C. The total run time for GC was 13 min/sample. The peaks were identified by comparing the retention time with the standard area. Calibration curves were plotted and standard showed R^2 value close to 0.999 was accepted.

Supplementary figures and tables

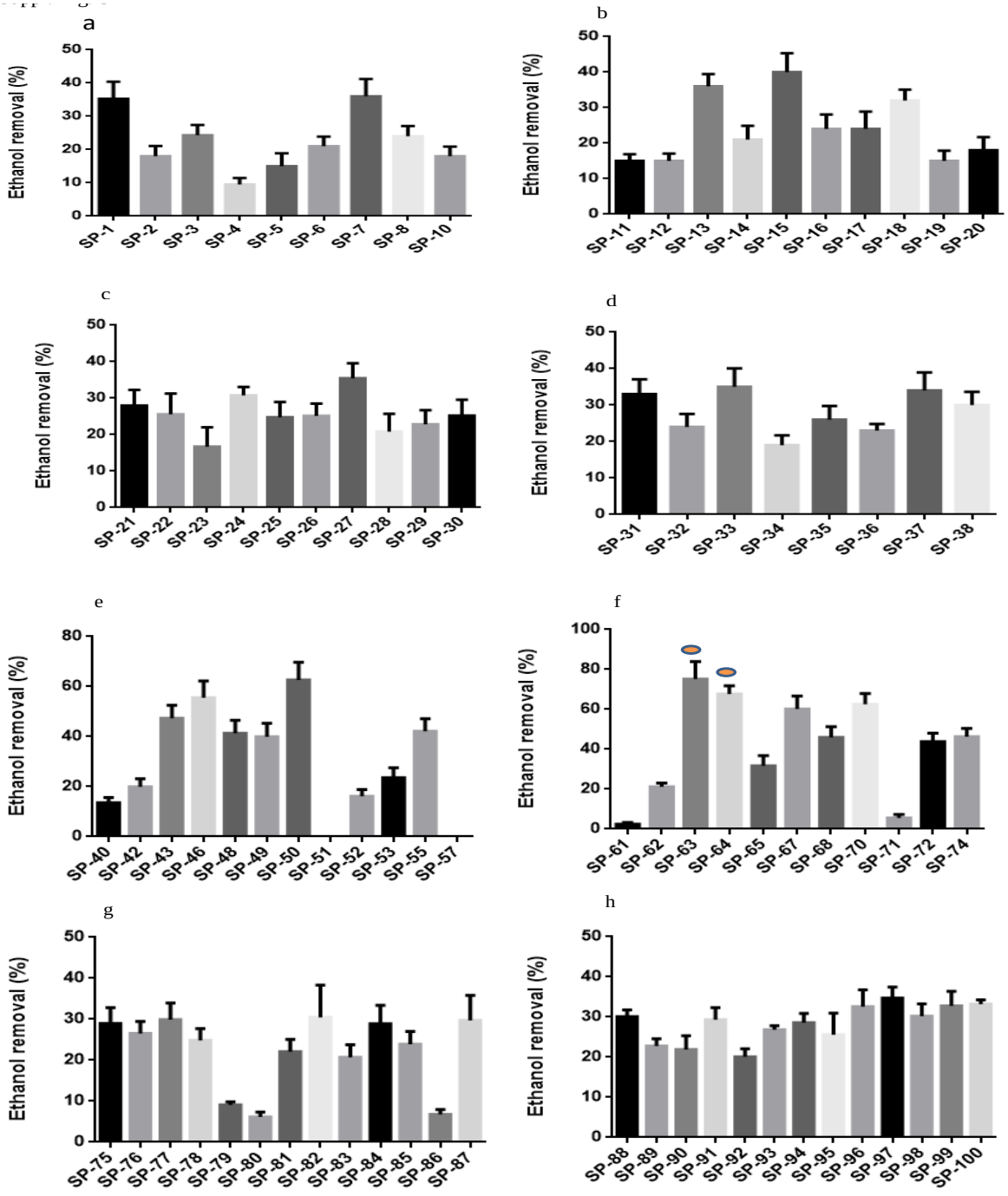
Figure S1



Colony PCR of transformants showing the full length 1.2 Kb ADH gene amplification (Lane 1-4) and small length amplification 750 bp (Lane 5-10). The selected *B. subtilis* transformants were screened by colony PCR with condition; 95 °C for 10 min, followed by 40 cycles of

denaturation at 95 °C for 15 sec, annealing at 55 °C for 15 sec, extension at 72 °C for 30 sec and a final elongation for 10 mins with the primers as given in suppl. Table S2.The PCR products were analyzed on 1% agarose gel and finally it was confirmed by sequencing.

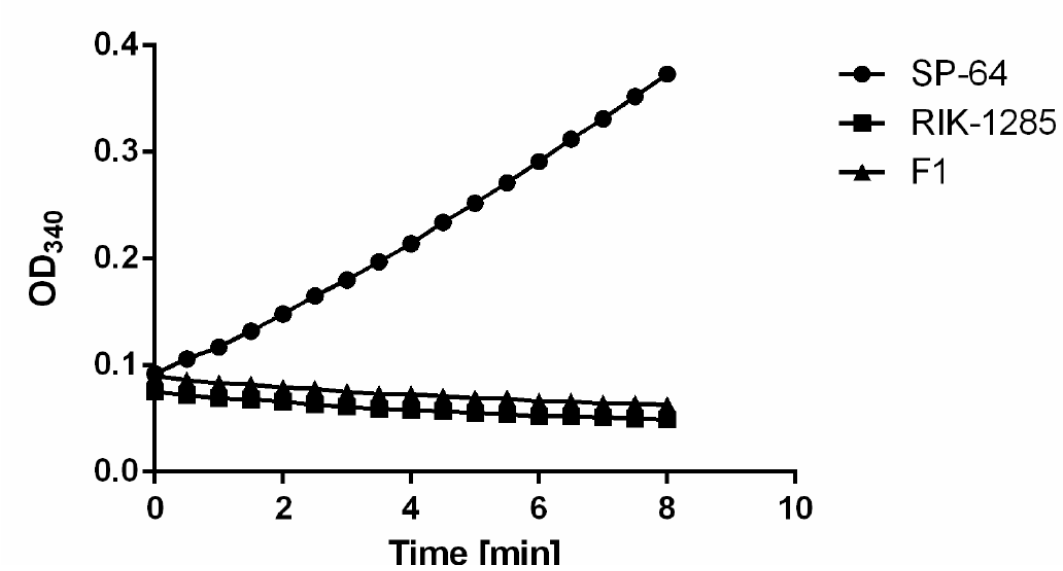
Figure S2



Ethanol removal measurements with signal peptide transformants. The signal peptide transformants were screened for ethanol removal ability. An equal amount of 1×10^7 cells of all transformants were inoculated into MM medium supplemented with ethanol (0.5%) and incubated for 24 h at 37 °C with shaking of 200 rpm. After the incubation period, each

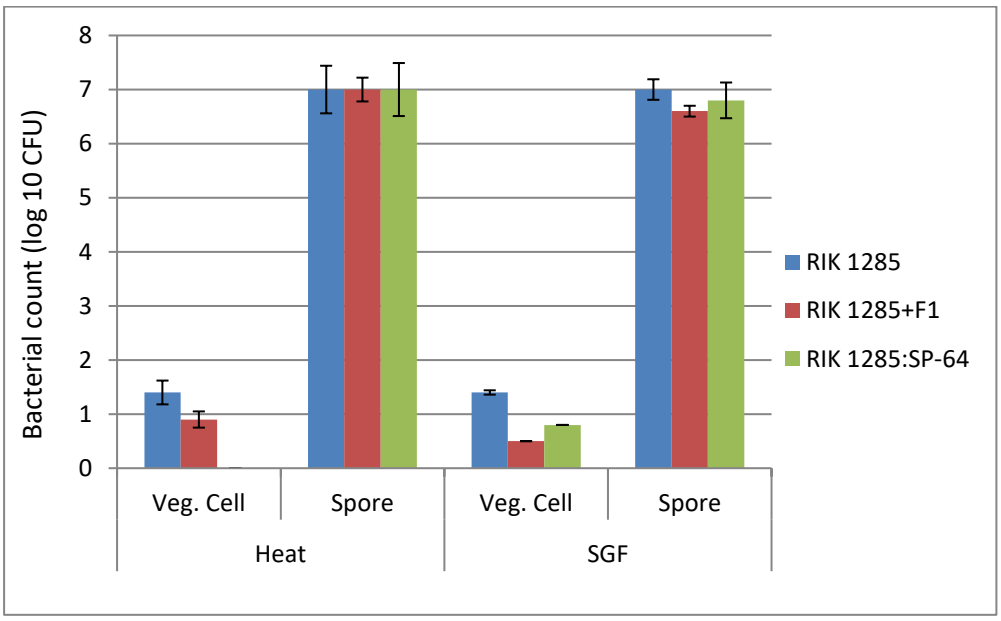
treatment's medium was filtered with 0.22 µm Millipore filtered and analyzed on GC-FID. This ethanol concentration is the common physiological concentration. All values are expressed as mean ± SEM (*n=3*).

Figure S3



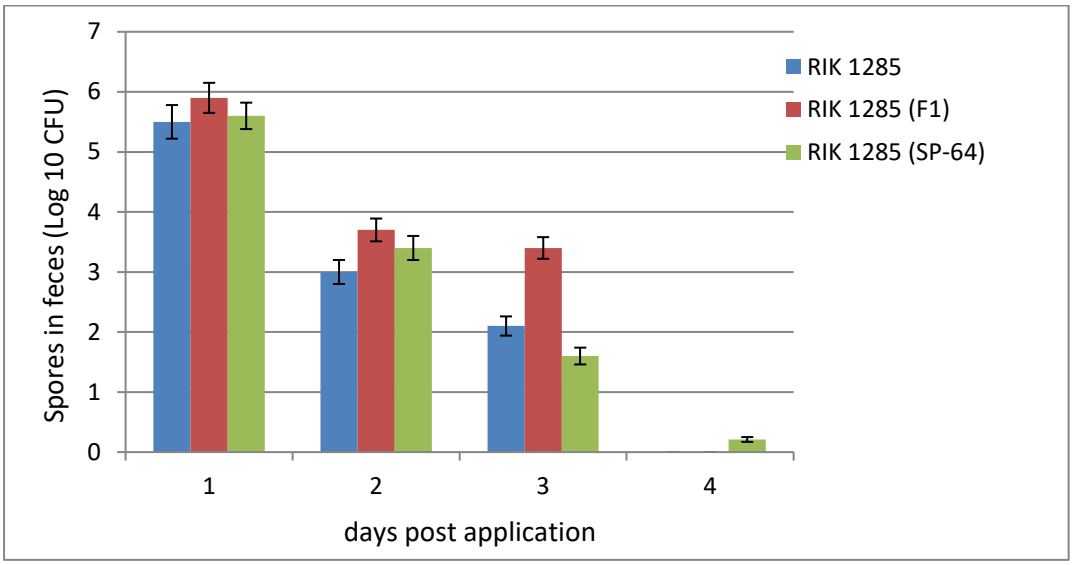
Observation of ADH-4 secretion in the culture supernatant by the *B. subtilis* RIK 1285, RIK 1285:ADH4 (F1) and RIK 1285:ADH4 (SP-64) by conversion of NAD to NADH. The increase in absorbance at 340nm(NADH) following oxidation of ethanol to acetaldehyde by the secreted ADH4.

Figure S4



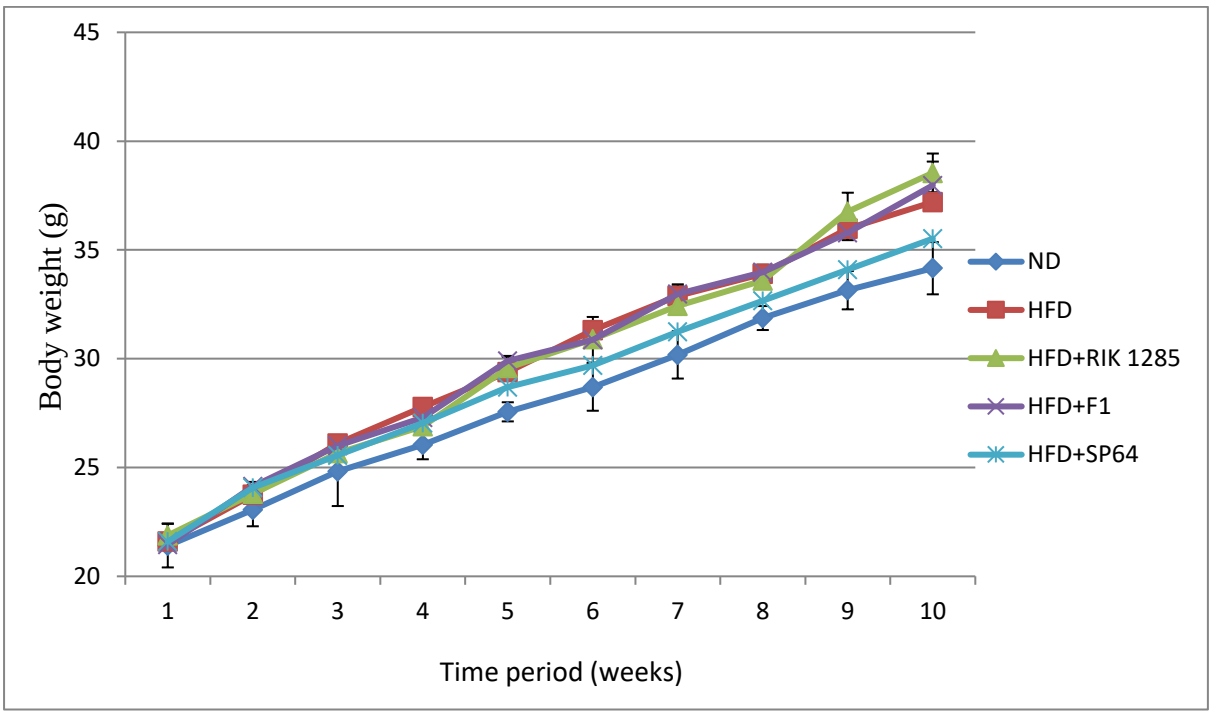
Spores survival test under heat and acidic conditions of simulated gastric fluid. Heat and SGF survival test of vegetative and spores of strains. All values are expressed as mean ± SEM (*n=3*).

Figure S5



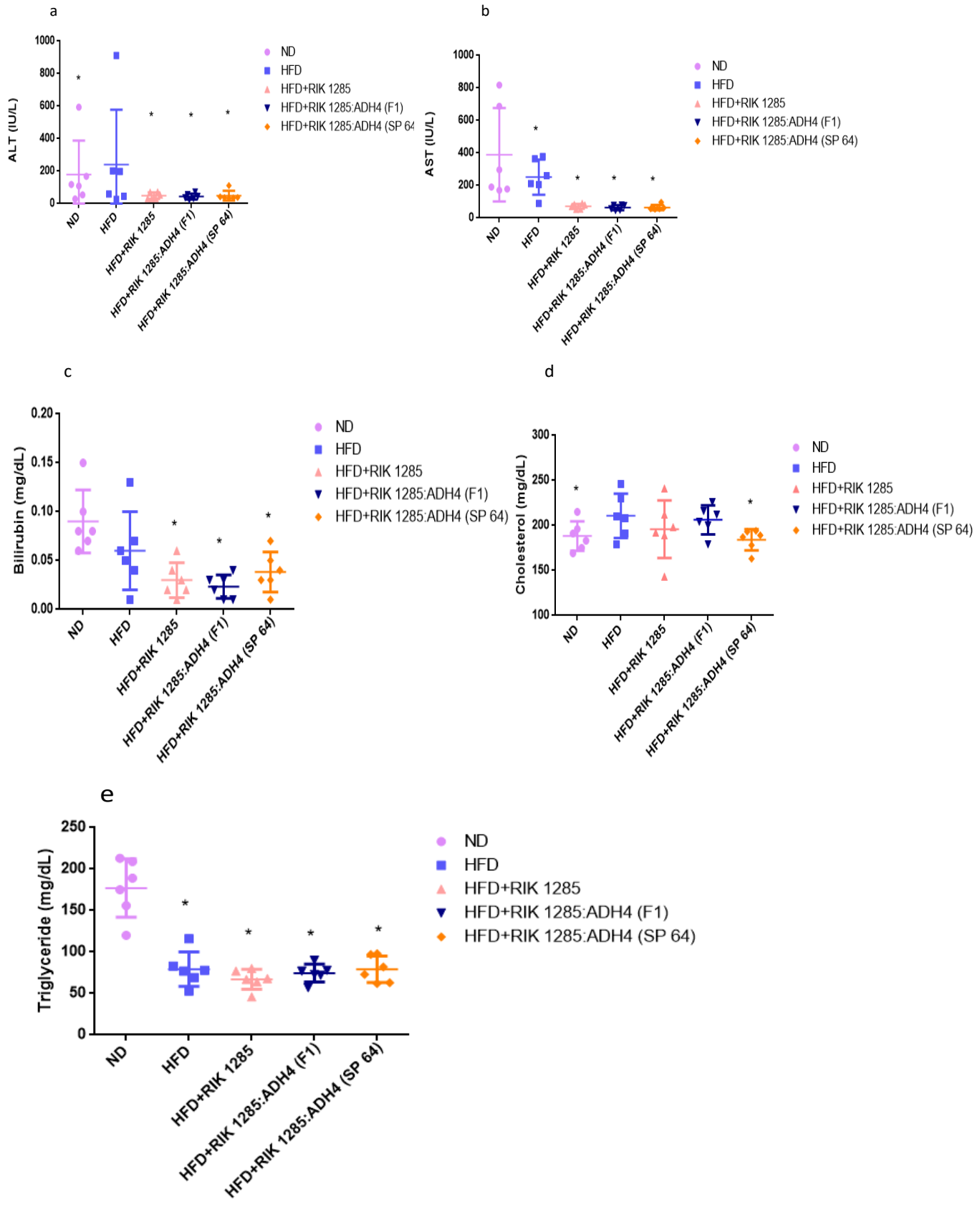
Test of colonization by the inoculated strains, Test of colonization by counting the spores populations in the feces. All values are expressed as mean ± SEM (*n=3*).

Figure S6



The changes in body weight throughout the 10 week experimental period under ND, HFD, HFD+ RIK 1285, HFD+ RIK 1285 (F1), HFD+ RIK 1285 (SP-64).

Figure S7


a
ALT (IU/L)
1000
800
600
400
200
0
ND
HFD
HFD+RIK 1285
HFD+RIK 1285:ADH4 (F1)
HFD+RIK 1285:ADH4 (SP 64)
b
AST (IU/L)
1000
800
600
400
200
0
ND
HFD
HFD+RIK 1285
HFD+RIK 1285:ADH4 (F1)
HFD+RIK 1285:ADH4 (SP 64)
c
Bilirubin (mg/dL)
0.20
0.15
0.10
0.05
0.00
ND
HFD
HFD+RIK 1285
HFD+RIK 1285:ADH4 (F1)
HFD+RIK 1285:ADH4 (SP 64)
d
Cholesterol (mg/dL)
300
250
200
150
100
ND
HFD
HFD+RIK 1285
HFD+RIK 1285:ADH4 (F1)
HFD+RIK 1285:ADH4 (SP 64)
e
Triglyceride (mg/dL)
250
200
150
100
50
0
ND
HFD
HFD+RIK 1285
HFD+RIK 1285:ADH4 (F1)
HFD+RIK 1285:ADH4 (SP 64)

Oral administration of engineered probiotic SP-64 improved the serum markers**. (a)** High-fat diet assumed to increase the alanine transaminase (ALT), that act as liver damage marker, however the level of ALT was found to be lower in bacterial treated groups **(b)** similarly, the level of aspartate transaminase (AST) was also lower in the bacterial treated groups. **(c**) lower level of bilirubin content was observed in bacterial treated groups **(d**) engineered SP-64 showed lower level of cholesterol under HFD treatments and it was comparable to ND groups. (f) triglyceride level was higher in ND group, as compared to bacterial treatments. (*n*=6 in each treatment). * $P < 0.05$ compared with the untreated group.

Table S1. Synthesized ADH4 gene sequence

ATGCATCATCATCATCATCATTCCAGCGGAGTGGATTTAGGAA
CGGAAAACCTGTATTTTCAGTCAATGGGCACGAAAGGCAAA
GTGATTAAATGTAAAGCAGCGATCGCTTGGGAAGCCGGCAA
ACCGCTTTGCATTGAAGAAGTCGAAGTTGCACCGCCGAAAG
CGCATGAAGTTAGAATCCAAATTATCGCTACATCACTGTGTCA
TACAGATGCCACAGTGATTGATAGCAAATTTGAAGGACTTGC
ATTTCCGGTGATCGTCGGCCATGAAGCTGCCGGAATTGTCGA
ATCAATCGGCCCGGGAGTTACAAATGTGAAACCGGGCGATA
AAGTTATTCCGCTTTATGCTCCGTTATGCAGAAAATGTAAATT
TTGCCTGTCTCCGCTGACAAATCTTTGTGGAAAAATCTCAAA
CCTGAAATCACCGGCCAGCGATCAACAGCTTATGGAAGATAA
AACAAGCCGCTTTACATGCAAAGGCAAACCGGTGTACCATTT
CTTTGGAACATCTACATTTTCACAATACACAGTTGTGAGCGAT
ATTAACCTGGCAAAAATCGATGATGATGCGAATCTGGAAAGA
GTTTGTCTGCTTGGCTGCGGATTTTCTACAGGCTATGGAGCA
GCGATTAATAACGCAAAAGTTACACCGGGCTCTACATGTGCG
GTGTTTGGCCTGGGCGGAGTCGGACTTTCAGCTGTTATGGG
CTGCAAAGCTGCCGGAGCCTCAAGAATCATCGGCATCGATAT
TAACAGCGAAAAATTTGTTAAAGCAAAAGCGCTTGGAGCTA
CAGATTGTTTAAACCCGCGCGATCTGCATAAACCGATTCAGG
AAGTCATTATCGAACTTACAAAAGGCGGAGTTGATTTTGCTT
TAGATTGCGCCGGCGGATCTGAAACAATGAAAGCAGCGTTA
GATTGTACAACAGCGGGCTGGGGAAGCTGCACATTTATCGG
AGTGGCTGCCGGCTCTAAAGGACTTACAGTCTTTCCGGAAG
AATTAATTATCGGCAGAACAATTAACGGCACATTTTTCGGCG
GATGGAAAAGCGTGGATTCTATCCCGAAACTGGTCACAGATT
ACAAAAACAAAAAATTTAACTTAGATGCACTGGTGACACATA
CACTGCCGTTTGATAAAATTAGCGAAGCGTTTGATCTTATGA
ACCAAGGCAAATCTATTCGGACAATTCTTATCTTT

Table S2

Primesr for cloning in pBE-S vector

ADHF- GGTGGTGGTACCATGCATCATCATCATCATCATTC

ADHR GGTGGTAAGCTTAAAGATAAGAATTGTCCGAATAG

Colony PCR primers

- SCRF-ATGCATCATCATCATCATCATTC
- SCRR-CCGAATAGATTTGCCTTGGTTC
- SCRRF-ACAGTGATTGATAGCAAATTTG
- SCRRR-TGTTGTACAATCTAACGCTGC

Identified signal peptide (PhoB)

ATGAAAAAATTTCCGAAAAAACTGCTGCCGATTGCGGTGCTGAGCAGCAT

TGCGTTTAGCAGCCTGGCGAGCGGCAGCGTGCCGGAAGCGAGCGCGG

Primers used for signal peptide identification

SPF- CAATAAATTCACAGAATAGTC

Table S3

Normal Diet (ND)	**High fat diet (HFD)**
Corn starch (500g)	Cellulose (65.5g)
Casein (210g)	Casein (265g)
Maltodextrine (100g)	Maltodextrine (160g)
Sucrose (39.15g)	Sucrose (90g)
Butter (24g)	Cholic acid (5g)
Lard (20g)	Lard (310g)
Soyabeen oil (20g)	Soyabeen oil (30g)
Cellulose (35g)	
Mineral mix (35g)	Mineral mix (51.4g)
Vitamin mix (15g)	Vitamin mix (21g)
L-methionine (3g)	L-methionine (4g)

Choline (2.75g)	Choline (5.0g)
BHT (0.014g)	BHT (0.014g)
DDW 500 ml	DDW 200 ml

Raw data

SE by group	stdev by group	mean PS6 by group	normeliezed mean PS6 by slide	mean PS6	subject number	Groups
1.11555	1.932188	30.00678	30.10577	28.076	4	1- WT
				28.134		
				20.085		
				19.884		
				18.572		
				21.453		
			31.88757	26.192	5	
				32.538		
				42.955		
				41.263		
				27.046		
				27.258		
				25.961		
			28.027	25.827	6	
				24.632		
				29.638		
				27.76		
				33.073		
				27.232		
1.941006	3.361921	39.99318	43.48321	22.438	11	2-HFD
				30.94		
				35.134		
				39.389		
				36.628		
				32.197		
			36.776	37.501	12	
				37.872		
				35.106		
				38.276		
				36.387		
				36.952		
				35.338		
			39.72033	43.57	13	

				32.255		
				37.006		
				33.727		
				45.867		
				45.897		
5.552766	9.617672	42.00001	31.73652	20.847	18	3-HFD+gavage
				21.888		
				18.974		
				30.317		
				21.128		
				19.778		
				24.319		
				27.067		
				28.242		
				26.743		
			43.45817	32.943	19	
				34.338		
				50.034		
				45.65		
				49.554		
				48.23		
			50.80533	47.491	20	
				36.616		
				48.737		
				56.748		
				45.743		
				69.497		
3.303021	5.721001	30.83546	37.36905	39.749	24	4-HFD+gavage+ADH
				23.906		
				20.34		
				27.4		
				29.492		
			28.41367	25.386	25	
				23.872		
				24.111		
				27.895		
				32.056		
				37.162		
			26.72367	28.33	26	
				25.406		
				20.396		
				32.918		

				33.373		
				19.919		
1.619383	2.804853	21.76737	19.01028	15.485	31	5-HFD+gavage+HDH secreted
				16.37		
				13.791		
				12.653		
				13.065		
				14.642		
			24.61767	22.051	32	
				22.165		
				23.398		
				28.984		
				22.126		
				28.982		
			21.67417	18.254	33	
				27.904		
				19.208		
				23.55		
				17.423		
				23.706		