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Optimization of Cocktail Composition and Up-scale Fermentation Process Development of Four Skin Commensal Bacterial Strains

(Optimalisasi Komposisi Koktail dan Pengembangan Proses Fermentasi Up-scale dari Empat Strain Bakteri Komensal Kulit)

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Abstract: Analyzing the actual microbial composition in the skin microbiome is riveting to create a potential source of active substances in skincare products. Our previous study isolated four bacterial strains from Javanese male and female skin samples, *Staphylococcus hominis* MBF12–19J, *Staphylococcus warneri* MBF02–19J, *Bacillus subtilis* MBF10–19J, and *Micrococcus luteu*s MBF05–19J. This study aimed to determine the composition of those strains in a bacterial cocktail and optimize the condition for up-scale laboratory production of the cocktail. To assess the ability of bacteria to coexist and live in cocktail communities, simultaneous monitoring was performed using the Deferred Growth Inhibition Assay (DGIA) method and real-time PCR for DNA copy number measurement. Results showed the best composition based on even distribution and cell growth viability was at the ratio 1.5:1:0.5:0.5 of *Micrococcus luteus* MBF05-19J, *Bacillus subtilis* MBF10-19J, *Staphylococcus warneri* MBF02-19J, *Staphylococcus hominis* MBF12-19J, which is equivalent to DNA copy number/mL 1.209 x 10^{24} CFU : 2.484 x 10^{41} CFU : 2.645 x 10^{41} CFU : 9.041 x 10^{35} CFU, respectively. The optimum growth incubation time of individual bacterial cultures for an up-scale 2-L bio-fermentor mixture was as follows; *Micrococcus luteus* MBF05-19J = 21 hrs; *Bacillus subtilis* MBF10-19J = 7 hrs; *Staphylococcus warneri* MBF02-19J = 17 hrs; *Staphylococcus hominis* MBF12-19 = 15 hrs. For up-scale conditions, the fermentation incubation time was 3 hours at 37°C, agitation 50 RPM, and aeration 5% dissolved oxygen.

Keywords: Skin microbiome, bacterial cocktail, growth curve, real time qPCR.

Abstrak: Menganalisis komposisi mikroba sebenarnya dalam mikrobioma kulit sangat menarik untuk menciptakan sumber potensial zat aktif dalam produk perawatan kulit. Penelitian kami sebelumnya mengisolasi empat strain bakteri dari sampel kulit pria dan wanita Jawa, *Staphylococcus hominis* MBF12–19J, *Staphylococcus warneri* MBF02–19J, *Bacillus subtilis* MBF10–19J, dan *Micrococcus luteus* MBF05–19J. Penelitian ini bertujuan untuk menentukan komposisi galur-galur tersebut dalam koktail bakteri dan mengoptimalkan kondisi untuk produksi koktail skala besar di laboratorium. Untuk menilai kemampuan bakteri untuk hidup berdampingan dan hidup dalam komunitas koktail, pemantauan simultan dilakukan menggunakan metode *Deferred Growth Inhibition Assay* (DGIA) dan *real-time* PCR untuk pengukuran jumlah salinan DNA. Hasil penelitian menunjukkan komposisi terbaik berdasarkan pemerataan dan viabilitas pertumbuhan sel adalah pada rasio 1,5:1:0,5:0,5 *Micrococcus luteus* MBF05-19J, *Bacillus subtilis* MBF10-19J, *Staphylococcus warneri* MBF02-19J, *Staphylococcus hominis* MBF12-19J, yang setara dengan nomor salinan DNA/mL masing-masing 1,209 x 10^{24} CFU; 2,484 x 10^{41} CFU; 2,645 x 10^{41} CFU ; 9,041 x 10^{35} CFU. Waktu inkubasi pertumbuhan optimum kultur bakteri individu untuk campuran skala bio-fermentor 2-L adalah sebagai berikut; *Micrococcus luteus* MBF05-19J = 21 jam; *Bacillus subtilis* MBF10-19J = 7 jam; *Staphylococcus warneri* MBF02-19J = 17 jam; *Staphylococcus hominis* MBF12-19 = 15 jam. Untuk kondisi up-scale, waktu inkubasi fermentasi adalah 3 jam pada suhu 37°C, agitasi 50 RPM, dan aerasi 5% oksigen terlarut.

Kata kunci: Mikrobioma kulit, bakteri koktail, kurva pertumbuhan, *real time* qPCR.

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INTRODUCTION

SKIN is an ecosystem of 1.8 m^2 of unique and diverse habitats that support various microorganisms to live[1]. The surface epithelium of the human body is involved in a reciprocal relationship with large and complex microbial populations. Resident microbiota is very diverse because multiple niches within the skin ecosystem differ in humidity, temperature, pH, antimicrobial peptide (AMP), and lipid content[2].

Many of these microorganisms are harmless and in some cases, have vital functions for humans. Symbiotic microorganisms occupy various recesses of the skin and protect the skin from invasion by pathogenic or harmful organisms[1]. The symbiotic relationship between skin microbiota and the host is a basic aspect for the exploration of skin microbes as a potential source to develop the active pharmaceutical ingredients for cosmetics or skin health[3].

Our previous study reported several commensal bacteria isolated from Indonesian young people's facial skin that have been characterized by 16S rRNA[4]. They are strain *Staphylococcus hominis* MBF12-19J; this bacterium is known as a commensal bacteria that can protect the skin from colonization of pathogenic bacteria by producing hominicin, an antibacterial molecule (5). Strain *Staphylococcus warneri* MBF02-19J; this has been reported to produce bacteriocin Nukacin ISK-1 and Warnericin RK, the first antibacterial peptide with activity against Legionella[6]. Strain *Bacillus subtilis* MBF10-19J; a bacterium that has been reported to produce Bacitracin on the skin surface, a toxin that helps fight Legionella[6]. The last is *Micrococcus luteus* MBF05-19J; research showed that this bacterium can produce DNA repair enzymes to prevent photo neoantigens in the skin due to UV rays[7].

In recent decades, microbial therapies including postbiotics have been widely studied and developed as a new class of active pharmaceutical ingredients (APIs)[8]. Generally Recognized as Safe (GRAS) bacteria can be developed to design a postbiotic bacterial cocktail in a preparation that provides benefits for facial skin health[9]. The resulting product has several advantages, such as containing GABA which has antioxidant properties[10] and is able to increase skin elasticity by regulating the expression of type I collagen[11]. Since the bacteria are initially the skin bacteria, it can minimize the risk of side effects.

However, the bacterial strains will compete to survive when grown together either through the production of antimicrobial compounds or nutritional competition[12] that can affect the actual proportion of the bacterial population in the cocktail fermentation. Therefore, optimizing the bacterial population in a specific concentration ratio is necessary to obtain the cocktail with an active and balanced composition.

The ability of bacteria to coexist and be viable in the cocktail community is simultaneously monitored by microbiological methods to analyze the viability of each strain through visual observations with the Deferred Growth Inhibition Assay, DGIA[13], and by a quantitative molecular method using real-time qPCR. Quantification with Real-Time qPCR is a common method to quantify bacteria based on the change in fluorescence proportional to the increase in the target, in addition to its ease of use and cost-effectiveness compared to other methods such as Southern Blot[14].

MATERIALS AND METHODS

MATERIAL. Bacterial Strains. Cryo-stock of *Staphylococcus hominis* MBF12–19J, *Staphylococcus warneri* MBF02–19J, *Bacillus subtilis* MBF10–19J, and *Micrococcus luteus* MBF05–19J were obtained from the collection of the Laboratory of Microbiology and Biotechnology, Faculty of Pharmacy, University of Indonesia isolated from our previous study[4].

The strain identities' as complete genome sequences are available in database (https://submit.ncbi.nlm.nih.gov/about/genome/), with accession numbers are as follows; *Staphylococcus warneri*: JAGMUP000000000; *Staphylococcus homini*: JAGMUC000000000; *Bacillus subtilis*: JAGMTL000000000; *Micrococcus luteus*: JAGMUB000000000.

Bacterial Growth Condition and Sub-culture. Strains were grown on their optimum media; *Staphylococcus hominis* MBF12-19J, *Staphylococcus warneri* MBF02-19J were streaked on Tryptic Soy Agar (TSA), while *Bacillus subtilis* MBF10-19J on Nutrient Agar, and *Micrococcus luteus* MBF05-19J on blood agar. All agar cultures were incubated aerobically at 37°C for 24 - 48 hours. For further sub-culture steps, single pure colonies were picked and re-streaked on new agar plates.

Further, strains were cultured into 9 ml of tryptic soy broth medium for aerobic enrichment for 24 h at 37°C without shaking. The individual cultures of strains in liquid medium were measured by referring them to Mc Farland 0.5 suspension, which is equal to Absorbance 0.1±0.05 and cell density $1.5x10^8$ CFU/mL, and as well by measuring the initial Optical Density (OD_{600}) of each individual culture.

Bacterial Viability Test. The individual broth culture of bacteria was measured at Optical Density (OD_{600}) 0.3±0.05 after incubation for 24 hours[13]. The bacterial suspension was loaded onto a filter paper 0.5 cm wide and affixed transversely on the Tryptic Soy Agar media surface after removing the excess by decantation. All preparations for DGIA were incubated

aerobically at 37°C for 24 hours[13]. The filter paper was then removed from the agar medium. The culture of indicator bacterium was loaded onto another filter paper of the same size and stuck perpendicular to the test bacteria lane, then incubated at 37°C for 24 hours aerobically. The indicator bacteria filter paper was then removed after incubating for 24 hours. The test preparations were re-incubated at various time variations, 6, 12, and 24 hours to see the zone of inhibition between bacteria. The inhibition zones formed were documented with a scanner and observations were made visually.

DNA Extraction. Presto™ Mini gDNA Bacteria Kit (with Lysozyme) (Geneaid, Taiwan) was used to extract all strains' genomic DNAs according to the manufacturer. All DNAs obtained were observed on agarose gel and visualized over a UV transilluminator. The concentration and purity of DNAs were measured using NanoDrop One Microvolume UV-Vis Spectrophotometer prior to use.

Realtime PCR. Analysis of proportion was carried out using real-time q-PCR and extrapolating the Ct value on each standard curve of strains, i.e., *Staphylococcus hominis* MBF12-19J, *Staphylococcus warneri* MBF02-19J, *Bacillus subtilis* MBF10-19J and *Micrococcus luteus* MBF05-19J. The proportion of strains was expressed in CFU log. The PCR mix and condition are presented in Table 2 and 3. Specific primer sets for Real-Time PCR were obtained by referring to the literature and re-examined using BLAST at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) The oligo primer pairs used are listed in Table 1.

The annealing temperature optimization was carried out using positive samples from each commensal strain. Optimal temperature parameters were determined based on primers that had previously been analyzed using software, i.e., at 55.5 °C, 57 °C, 58.5°C, and 60°C, respectively.

Bacterial Cocktail Fermentation. Prior to up-scale fermentation of bacterial cocktail, each bacterium was grown in TSB production media, then OD_{600} was measured every hour until it reached a stationary phase to obtain the growth profile of each bacterium in a larger volume of culture. The bacterial cocktail was mixed after each bacterium was individually cultured - rejuvenated according to their respective growth curves and the optimum OD value based on a calculated ratio mixture. Samples were taken at 1, 2, and 3 hours to check the density of bacteria by measuring OD_{600}. Based on the optimum growth profile, the bacterial cocktail was cultured in a fermenter by applying the best incubation time for the cocktail.

RESULTS AND DISCUSSION

Bacterial Viability. The result of the competition as observed visually is presented in Figure 1. After 12 hours of incubation, the commensal strain was estimated to produce the optimum amount of antimicrobial peptides so that the inhibitory activities between the indicator strains have yet appeared. At 24 hours, the bacteria were overgrown. This could probably be due to the high concentration of bacteria

Table 1. Specific primer sequences used for real-time PCR quantification.

Oligoprimer	Sequences	Reference
ShomF	5' – TAGATGGATCTGAAACAGTAGTAT-3'	[15]
ShomR	5'-CCTTCAACAATACCAAATTCGTC-30'	
SwarF	5'-TGTAGCTAACTTAGATAGTGTTCCTTCT-3'	[16]
SwarR	5'-CCGCCACCGTTATTTCTT-3'	
BsubF	5'-CGCCGTTTACGTTCTGTAGGC-3'	[17][18]
BsubR	5'-CGGTTTAAGCCGTATGGAGCG-3'	
MlutF	5'-GGGAGGAGACGTGGACGAAG-3'	[19]
MlutR	5'-AGTACGAGGCCGTGAACGTG-3'	

Table 2. q-PCR reagent setting.

Reagen	Volume	Final Concentration
Nucleus Free Water (NFW)	6.7 µL	
THUNDERBIRD SYBR™ qPCR Mix	10 µL	
Forward Primer	0.6 µL	0.3 µM
Reverse Primer	0.6 µL	0.3 µM
50X ROX Reference dye	0.1 µL	0.1X
DNA Solution	2 µL	
Total	**20 µL**	

[Source : THUNDERBIRD SYBR® Green Based Quantitative PCR, Toyobo, Japan]

Table 3. q-PCR cycle setting.

Cycle	Temperature (°C)	Time (s)	Repetition
Predenaturation	95	60	1x
Denaturation	95	15	40x
Annealing	55.5 - 60	15	
Extension	60	60	

[Source : BioMetra T-Optical Thermal Cycler PCR, Analytik Jena, Germany]

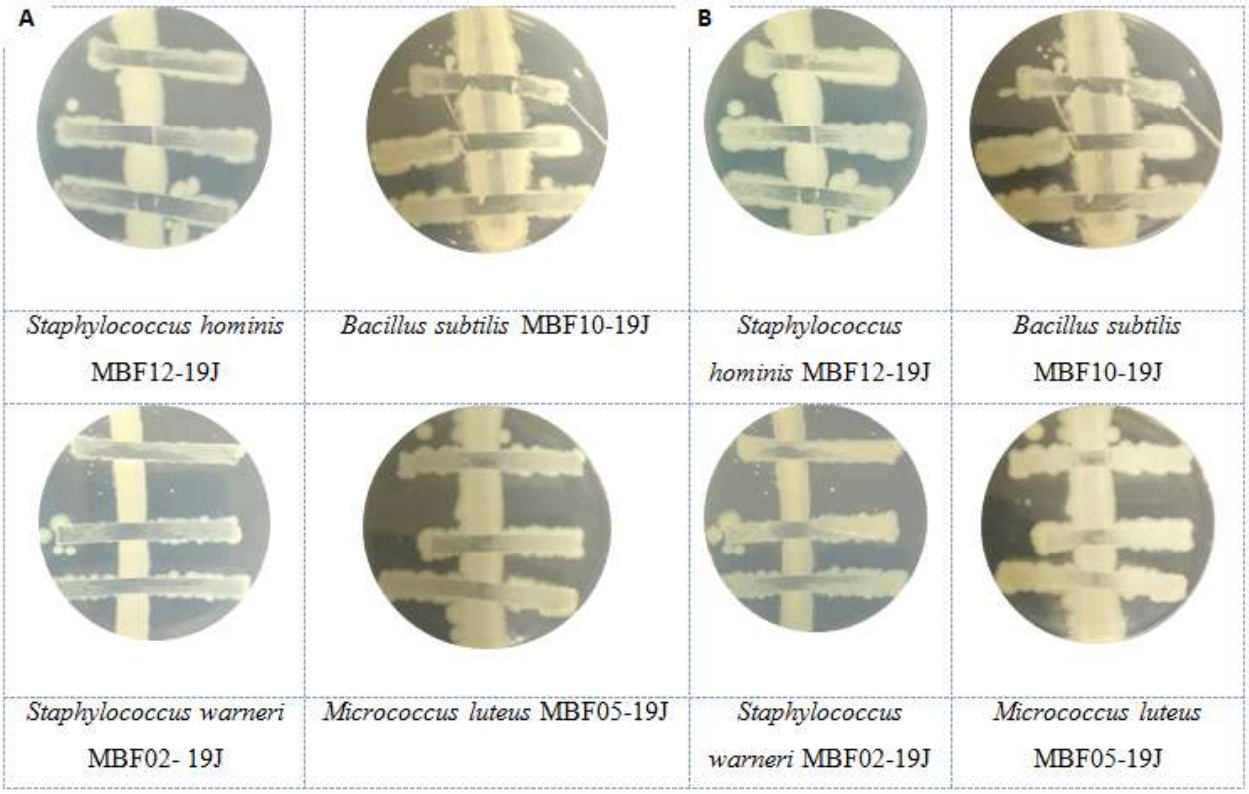

Figure 1. DGIA bacterial competition result: the test bacterium (straight crossing the agar) against the indicator strains. (A) After 6 hours of incubation time, (B) 12 hours of incubation, the zone of inhibition between commensal probiotic strains was more clearly visible.

inoculation used. The viability test results between commensal bacteria showed consistent results: the competition between microbes in fighting for nutrients from Tryptic Soy Agar media and the increasing incubation time.

Further, the proportion of commensal strains in a bacterial cocktail was analyzed with incubation time parameters of 0, 2, 4, and 6 hours using Real-Time PCR. Bacteria cocktail composition design is listed in Table 4. The results are presented in Table 5.

Table 4. Bacteria cocktail composition design.

Composition	Commensal Bacteria			
	M. luteus MBF05-19J	*B. subtilis* MBF10-19J	*S. warneri* MBF02-19J	*S. hominis* MBF12-19J
K1	1	1	0.5	0.5
K2	1.5	1	0.5	0.5
K3	1	1.5	0.5	0.5
K4	1.5	1.5	0.5	0.5

Analysis of Composition and Optimal Conditions of Bacterial Cocktails. The proportion of those strains was analyzed by extrapolating the Ct value on each standard curve of commensal strains. The proportion of commensal strains was expressed in the CFU log. The results of Real-Time PCR of bacterial cocktails at various incubation times are presented in Table 5. The most proportional cells of all strains in the cocktail are at incubation time 4 hrs consisting of 1.209×10^{24} CFU; 2.484×10^{41} CFU; 2.645×10^{41} CFU; 9.041×10^{35} CFU of respective strains.

Table 5. Real-time PCR of bacterial cocktail results at various incubation times.

	Ct 1	Ct 2	Ct *mean*	Log CFU	CFU
Micrococcus luteus MBF05 – 19J					
0	12.97	12.93	12.95	23.11790	1.311×10^{23}
2	12.75	12.68	12.715	23.44643	2.795×10^{23}
4	12.13	12.39	12.26	24.08252	1.209×10^{24}
6	18.01	18.42	18.215	15.75743	5.720×10^{15}
Staphylococcus hominis MBF12 – 19J					
0	13.03	13.09	13.060	36.26444	1.838×10^{36}
2	12.69	12.60	12.645	37.86538	7.328×10^{37}
4	11.77	11.69	11.730	41.39518	2.484×10^{41}
6	12.26	12.06	12.160	39.73637	5.449×10^{39}
Staphylococcus warneri MBF02 – 19J					
0	12.40	12.44	12.420	36.71460	5.183×10^{36}
2	12.22	12.18	12.200	37.13564	1.366×10^{37}
4	9.90	10.02	9.960	41.42255	2.645×10^{41}
6	13.57	13.68	13.625	34.40848	2.561×10^{34}
Bacillus subtilis MBF10 – 19J					
0	14.15	14.24	14.195	29.28240	1.916×10^{29}
2	12.71	12.25	12.480	31.90154	7.971×10^{31}
4	9.62	10.03	9.825	35.95626	9.041×10^{35}
6	18.26	18.90	18.580	22.58562	1.368×10^{22}

The best composition based on even distribution of cell growth is in K3, *Micrococcus luteus* MBF05-19J: *Bacillus subtilis* MBF10-19J: *Staphylococcus warneri* MBF02-19J: *Staphylococcus hominis* MBF12-19J 1.5: 1: 0.5: 0.5 at incubation 4 hours as shown in Fig. 2 and Fig. 3. The results of the quantification of K3 composition which are equivalent

to the number of bacteria according to the number of DNA copies/mL are 1.209 x 10^{24} CFU; 2.484 x 10^{41} CFU: 2.645 x 10^{41} CFU; 9.041 x 10^{35} CFU.

Growth Profile and Fermentation Yield. The individual growth profile strains showed the optimum time as follows; i.e., *Micrococcus luteus* MBF05-19J = 21 hrs; *Bacillus subtilis* MBF10-19J = 7 hrs; *Staphylococccus wareri* MBF02-19J = 17 hrs; *Staphylococcus hominis* MBF12-19 = 15 hrs as shown in Fig. 4.

By applying the growth profile, the best results for bacterial cocktail fermentation that were up-scaled are as follows, incubation time of 3 hours at 37°C, agitation 50 RPM, and 5% dissolved oxygen aeration, with yield presented in Table 6. The 3 hrs incubation yield showed a decline, but it is the most sufficient time for all strains to co-exist and interact in the cocktail fermentation. Hence, for up-scale fermentation, the 3 hrs incubation time is presumed to be the best condition for cocktail fermentation time.

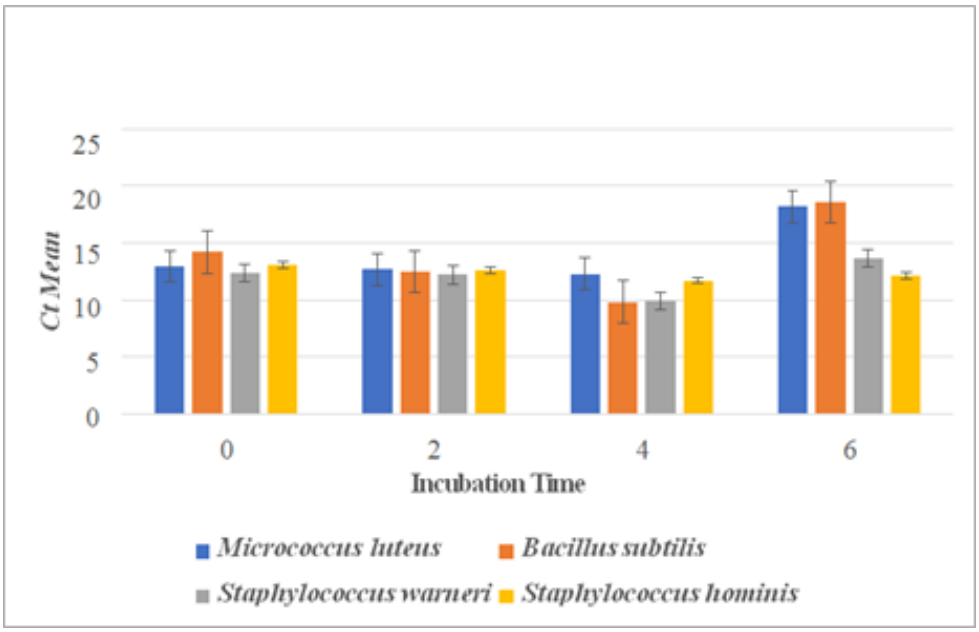

Figure 2. Ct mean of four strains in bacterial cocktails at various incubation times (hour).

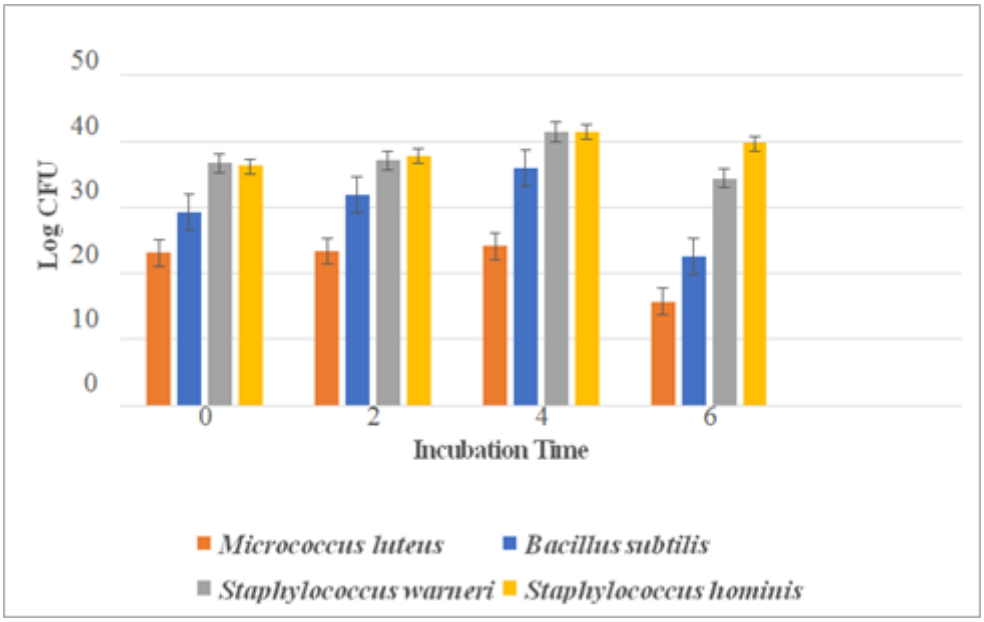

Figure 3. Commensal strain composition in bacterial cocktails at various incubation times (hour).

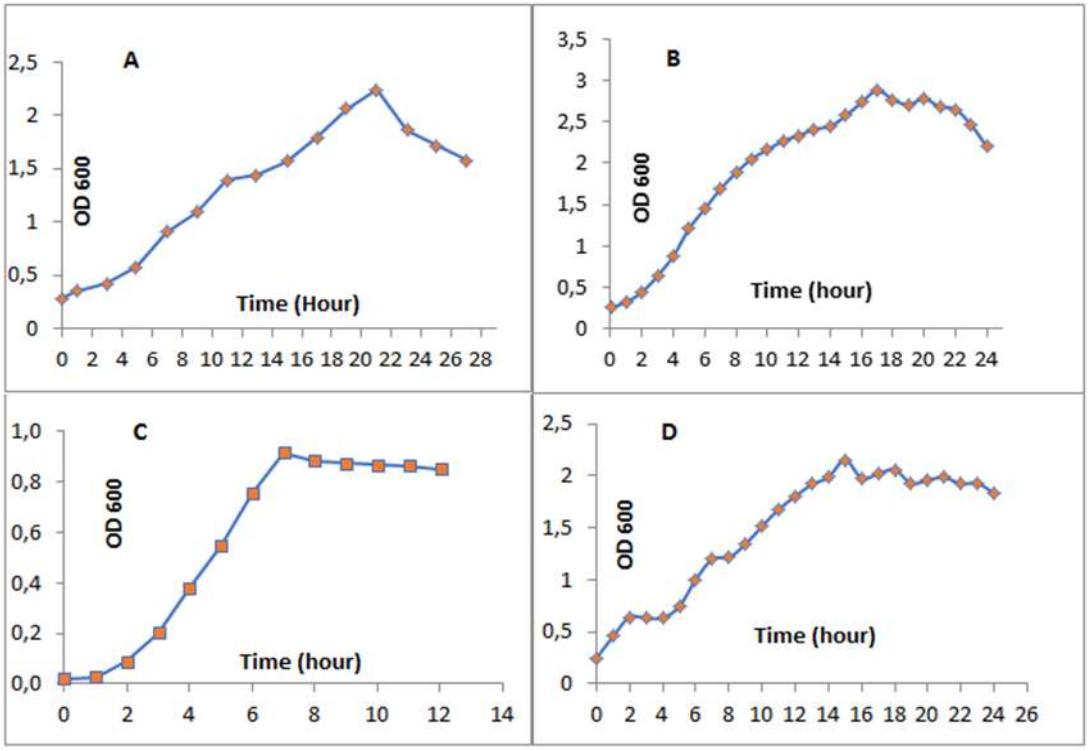

Figure 4. Bacterial growth curve. (A) *Micrococcus luteus* MBF05-19J with an optimum time of 21 hrs, (B) *Bacillus subtilis* MBF10-19J with an optimum time of 7 hrs, (C) *Staphylococcus warneri* MBF02-19J with an optimum time of 17 hrs, (D) *Staphylococcus hominis* MBF12-19J with an optimum time of 15 hrs.

Table 6. Results of bacterial cocktail density based on OD_{600} values at various incubation times.

Sampling hour	Bacterial Cocktail Density (OD_{600})				
	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
1	3.56	3.20	3.34	3.15	3.42
2	3.50	3.19	3.31	3.13	3.17
3	3.30	3.18	3.15	3.10	3.15

CONCLUSION

The best bacterial composition based on even distribution and cell growth viability was *Micrococcus luteus* MBF05-19J; *Bacillus subtilis* MBF10-19J; *Staphylococcus warneri* MBF02-19J; *Staphylococcus hominis* MBF12-19J at the ratio 1.5:1:0.5:0.5, which equivalent to DNA copy number/mL 1.209 x 10^{24} CFU; 2.484 x 10^{41} CFU; 2.645 x 10^{41} CFU; 9.041 x 10^{35} CFU, respectively at 4 hrs incubation. The optimum growth incubation time of individual bacterial cultures are as follows; *Micrococcus luteus* MBF05-19J = 21 hours; *Bacillus subtilis* MBF10-19J = 7 hrs; *Staphylococcus warneri* MBF02-19J = 17 hrs; *Staphylococcus hominis* MBF12-19 = 15 hrs, respectively, with condition for an up-scale 2-L bio-fermenter of cocktail is 3 hours at 37°C, agitation 50 RPM, and aeration 5% dissolved oxygen.

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