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Isolation and Identification Probiotic Candidates Bacteria from Crossbreed Fries Holland (CFH) Colostrum That Grown on Nutrient Agar with Olive Oil and Casein Enrichment

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Abstract: Aims of the research were to isolate the bovine colostrum indigenous bacteria that potent as probiotics with Nutrient Agar (NA) as a growth media. Selected isolate expected to develop as anti-pathogen in food spoilage and digestion. Material used including bovine colostrum taking from lactating Crossbreed Fries Holland (CFH) 1-3 days after partus which done compositely. Crossbreed Fries Holland colostrum everyday was taken 250 mL at the same time in the morning and afernoon and then stored at the refrigerator. Research done with qualitative method which analyzes descriptively through isolation step cover: instrument sterilization, dilution, morphology observation and pure culture production, then continued with identification step (gram staining, catalase test, oxidase test, low pH, high bile salt and API Test). Result showed that isolation with NA with olive oil addition obtained 10 bacteria (5 *Bacillus* genus, 3 *Micrococcus* genus and *Staphylococcus* genus). While at the NA with 1% casein addition obtained 12 pure isolate including: 2 *Staphylococcus* genus, 5 *Bacillus* genus, 2 *Micrococcus* genus and each *Streptococcus*, *Enterobacter* and *Veillonella* was found 1. Catalase and oxidase test resulting in two groups that cover *Bacillus* and *Coccus* group. Twenty isolate identified as gram positive bacteria, while two genus such as *Enterobacter* and *Veillonella* was identified as gram negative bacteria. Gram positive bacteria tested to determine the species using API test and resulting two dominant bacteria species with code Ic8 for *Bacillus coagulans* species with 93.7% similarity, Ic12 for *Aneurinibacillus aneurinilyticus* with 99.1% similarity.

Keywords: Bovine colostrum, identification, isolation, NA, probiotic candidate

INTRODUCTION

Microrganisms in dairy milk or bovine colostrum were vary in type and amount, one of it's type is bacteria which harmfull or favorable. Favorable bacteria that almost exist in dairy milk or bovine collostrum is *Coccus*, *Bacillus* and *Lactobacillus* groups of Lactic Acid Bacteria (LAB) which has a potential to be a probiotic. Probiotic bacteria is favorable microorganisms that would give positive health effects by controlling gastrointestinal microflora balance. The bacteria will multiply inside the gastrointestinal tract and give health effects, not pathogenic and non toxic.

Majority of probiotic bacteria was LAB groups that could produce organic acid, bacteriocin, peroxide which role in detain or even kill the pathogen bacteria. Lactic acid bacteria naturally occupy two ecology systems, that is food products and human gastrointestinal tract. In 1980's LAB was develop into 15 genus but only 5 genus that found in fermented milk products i.e., *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp. and *Streptococcus* sp. The LAB is a natural contaminant in milk products, it is because the main substrate was lactose so that known as milk bacteria (Surono, 2004; Rahayu, 2008).

Beside milk, colostrum is lactose based material that contain 90 bioactive compound and also role as source of probiotic bacteria. Colostrum is initial foods that could help mammals baby survive at the new environment and a yellowish liquid which contain 10-17 times lymphocyte, monocyte, neutrophils, protein and antibodies from mature milk (Frandson, 1992). Dairy milk and bovine colostrum contained antimicrobial compound such as lactoferin, lactoperoxidase, lactoglobulin and lactolipid that include lactenin groups. Beside that, less than 20.000 cfu/mL of bacterial colony and less than 100 cfu/mL coliform found in colostrum (Frandson, 1992; Naidu, 2000; Paikkanen and Aalto, 2000; Heinrichs and Coleen, 2011). Availability of antibodies, nutrients rich, antimicrobial activities has invite the functional

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microorganisms such as *Lactobacillus* and *Bifidobacterium* genus which used widely as probiotics LAB.

Lactic acid bacteria isolated from colostrum was 55.3% *Lactobacillus* genus which consist of 64% *L. plantarum*, 16% *L. fermentum* and 9% *L. pentosus*. Twenty eight percent of strain isolated from human colostrum shown resistance to pH of gastrointestinal tract and high bile salt concentration. While the LAB that isolated from bovine colostrum could produce hydrogen peroxide, diacetyl, aldehyde and bacteriocin which is bactericidal towards gram positive and negative bacteria, so that used as probiotics (Collado *et al*., 2009; Dubos *et al*., 2011).

Specific media will needed to make grow selected probiotic LAB strain, especially isolated from bovine colostrum. Nutrient Agar used as general media with addition of olive oil and casein as enrichment, which role as fat and protein sources and also gave high nutrient characteristics as same as bovine colostrum. The research objective was to isolate bovine colostrum bacteria that potential as probiotics which grown at enriched NA, therefore could be used as anti-pathogen agent in food spoilage and gastrointestinal tract.

MATERIALS AND METHODS

Colostrum taken compositely morning and afternoon from lactating CFH at 1-3 days after partus at farmer group KUBE PSP Maju Mapan, then stored at the refrigerator. Isolation of bacteria was done using enriched NA with composition 28 g NA in 1 L aquadest and two different enrichment i.e., 1% casein and olive oil. Bacterial colony appears purified and identified with gram staining, catalase test, oxidase test, low pH test, high bile salt test (2%) and API test (Fardiaz, 1993; Ruzanna, 2011; Safitri, 2011).

RESULTS AND DISCUSSION

Total bacteria colony of CFH colostrum: Result (Table 1) showed that average total bacteria colony of CFH colostrum was $21.3\times10^8\pm5.72\times10^8$ cfu/mL and it's increase in line with milking days, while higher total bacteria colony shown by composite samples. The increase of total bacteria colony happen at different milking days because the decrease of antimicrobial compound of CFH colostrum (Khusnul, 2013). Colostrum antimicrobial compound such as lactoferin was high at 24 h after partus and then decrease in line with the increase of milking days (Rahman, 2010; Khotimah and Pujo, 2013). The antimicrobial activity inside the colostrum getting lower so that the total bacteria colony increased. Meanwhile, higher total bacteria colony in mix samples resulted by storage time and temperature. Six days storage in refrigerator temperature gave chance for microorganisms to grow.

Isolation CFH colostrum bacteria on enriched NA: Thirteen bacteria isolate grown at NA media with olive oil enrichment was isolated from CFH colostrum. Identification of isolated bacteria resulting in 5 bacteria isolate of *Bacillus* sp., 3 bacteria isolate of *Micrococcus* and 2 genus. Olive oil addition function as fat source on NA media, meanwhile it can prevent carbohydrate fermentation that happen which could detain proteolytic bacteria growth (Pelczar, 2005; Ray, 2001). Bacteria that grow majority derived from mixture (composite) CFH colostrum samples and isolated 10 purified bacteria on slope agar then identified biochemically (Table 2). Staining gram showed that 10 bacteria isolate was gram positive bacteria which could maintain dark purple of metal color after it wash with alcohol (Feliatra *et al*., 2004). Catalase test was done to determine aerob, anaerob or facultative anaerob bacteria, with all the bacteria was positives. Oxidase test determine enteric bacteria which resulting 7 non enteric and 3 enteric bacteria.

Bacteria colony isolated on NA media with 1% casein enrichment was 12 bacteria isolate then identified by morphology identification, gram staining, catalase and oxidase test. The result shown six genus found. i.e., *Bacillus*, *Micrococcus*, *Streptococcus*, *Staphylococcus*, *Enterobacter* and *Veillonela*. As gram positives bacteria 5 *Bacillus*, 2 *Micrococcus*, 1 *Streptococcus*, 2 *Staphylococcus* was found with *Enterobacter* and *Veillonela* also identified as gram negatives bacteria. *Bacillus* identified as bacil form bacteria with 0.3-2.2×1.27-7.0 µm length, gram positives, catalase positives, oxidase negatives and morphology type of white or yellowish color, flat or irregular fringe with convex surface (Pelczar, 2005). Others gram positives bacteria isolated was *Micrococcus* which have circular form, 0.5-3.5 µm diameter, catalase positives with morphology type of

Table 1: Total bacteria colony of CFH colostrum

	Repetition ($\times10^8$)			
Milking day	1	2	Total (cfu/mL)	Average (cfu/mL)
Day 1	15.30	17.37	32.67	16.34
Day 2	17.89	21.16	39.05	19.53
Day 3	22.92	23.52	46.44	23.22
Mix (composite)	27.72	24.49	52.21	26.11
Average total bacteria colony				21.30×10^8

Table 2: Characteristic of bacteria isolated from CFH colostrum grown at NA with olive oil enrichment

			Test		
Code	Isolate character	Gram stain	Catalase	Oxidase	Genus prediction
Ik1	Yellow egg flat fringe convex surface	Small, bacil, spores gram +	+	-	*Bacillus* sp.
Ik2	Yellow egg flat fringe flat surface	Coccus, separated gram +	+	-	*Micrococcus* sp.
Ik5	Clear white flat fringe flat surface	Small, bacil, fat, spores	++	--	*Bacillus* sp.
Ik6	Creamy milky white flat fringe convex surface	Coccus, separated gram +	+	+	*Staphylococcus* sp.
Ik7	Milky white irregular fringe convex surface	Coccus, separated, clustered gram +	+	-	*Staphylococcus* sp.
Ik8	Yellow egg flat fringe convex surface	Tetra coccus gram +	+	+	*Micrococcus* (tetragenous)
Ik9	Yellowish white flat fringe convex surface	Small, bacil, fat, spores gram +	+	-	*Bacillus* sp.
Ik10	Milky white flat fringe convex surface	Small, bacil, short, spores gram +	+	+	*Bacillus* sp.
Ik12	Dark yellow flat fringe covex surface	Coccus, separated gram +	+	-	*Micrococcus* sp.
Ik18	Clear white irregular fringe flat surface	Bacil, spores gram +	+	-	*Bacillus* sp.

Table 3: Characteristic of bacteria isolated from CFH colostrum grown at NA with 1% casein enrichment

			Test		
Code	Isolate character	Gram stain	Catalase	Oxidase	Genus prediction
Ic1	White irregular fringe convex surface	Bacil, spores gram +	+	-	*Bacillus* sp.
Ic2	White irregular fringe flat surface	Coccus, separated gram +	+	-	*Staphylococcus* sp.
Ic3	White flat fringe flat surface	Coccus, chain	-+	--	*Streptococcus* sp.
Ic5	Milky white irregular fringe flat surface	Coccus, separated, concentrated gram +	+	-	*Staphylococcus* sp.
Ic7	Yellow irregular fringe convex surface	Coccus gram +	+	+	*Micrococcus* sp.
Ic8	White irregular fringe convex surface	Bacil, spores gram +	+	-	*Bacillus* sp. Megaterium
Ic11	White irregular fringe convex surface	Bacil, no spores gram -	+	-	*Enterobacter*
Ic12	Milky white flat fringe convex surface	Bacil, spores gram +	+	-	*Bacillus* sp.
Ic13	Yellowish white irregular fringe convex surface	Bacil, spores gram +	+	-	*Bacillus* sp.
Ic17	White flat fringe flat surface	Bacil, spores gram +	+	-	*Bacillus* sp.
Ic20	White flat fringe flat surface	Coccus gram -	+	-	*Veillonella*
Ic21	Yellowish white irregular fringe convex surface	Coccus gram +	+	-	*Micrococcus* sp.

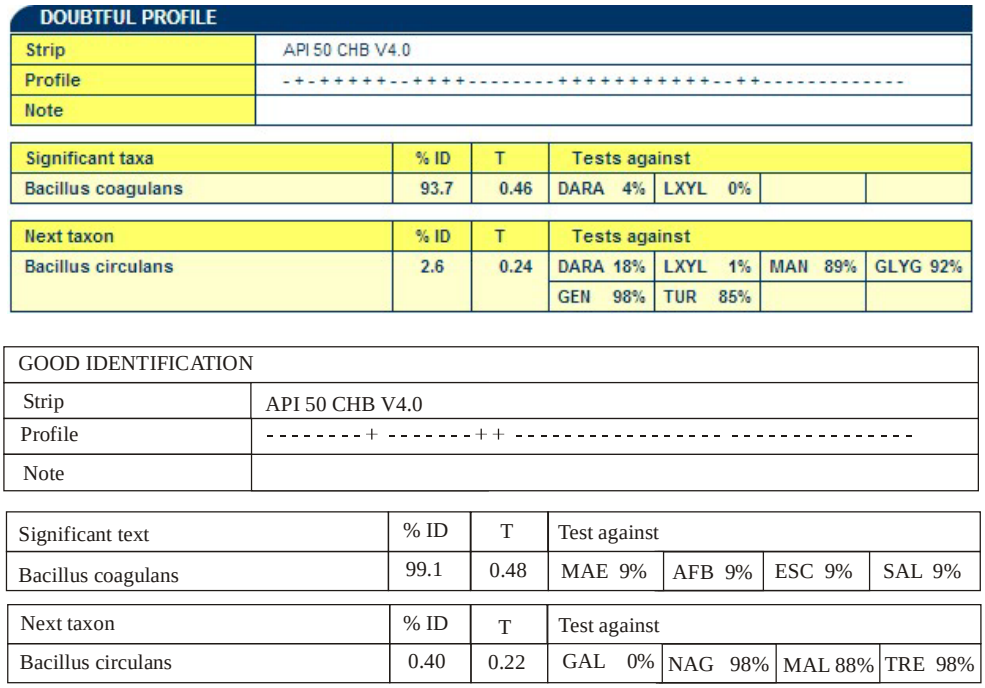
white or yellowish color, irregular fringe and convex surface. *Streptococcus* and *Staphylococcus* also have circular form and gram positives, but *Staphylococcus* was catalase positives, has 0.5-1,5 µm diameter, single or paired, irregular concentrated. While *Streptococcus* has circular form, less than 2 µm diameter, chained, gram positives, catalase negatives with morphology white color, flat fringe and flat surface (John *et al*., 1984; Fardiaz, 1993; Ray, 2001; Pelczar, 2005).

Enterobacter and *Veillonella* is gram negatives bacteria, which is harmfull bacteria with circular form, catalase positives, non-motil and no spores. This two bacteria identified as contaminant of CFH colostrum samples, because the bacteria was not came from the CFH colostrum. Generally bovine colostrum contain favorable probiotic bacteria that could give positive health effects (Finamore *et al*., 2012). Beside both bacteria, *Micrococcus* also identified as CFH colostrum contaminant and include milk spoilage bacteria that could hydrolyze protein into amino acid and degrade fat with lipase enzyme so that milk goes slimy (Ray, 2001; Vimont *et al*., 2006).

Bacteria isolated from CFH colostrum that grown at enriched NA with olive oil and 1% casein addition (Table 3) dominated by *Bacillus* sp. genus. Continue with that, API test 50 CHB was done (Fig. 1) and the result gave 2 dominant species with code Ic8 for *Bacillus coagulans* with 93.7% similarity and Ic12 for *Aneurinibacillus aneurinilyticus* with 99.1% similarity.

Low pH and bile salt test for selected CFH colostrum bacteria isolate: Selected bacteria grown in media that has low pH (pH 4) and high bile salt concentrations (2% bile salt). Low pH and high bile salt screening was done to determine survival ability of selected bacteria at gastrointestinal tract as requirement for probiotic bacteria candidates. Positive result, shown by muddy and deposition form on Nutrient Broth with pH 4 and 2% bile salt concentrations as growth media.

The result showed that CFH colostrum were potential as source of LAB probotic candidates that are gram positive bacteria with ability in producing organic acid, bacteriocin, peroxides and antimicrobial compounds. Lactic acid bacteria needs complex nutrients, therefore the habitat should be nutrient rich such as milk, meat, drinks or vegetables. Crossbreed Fries Holland Colostrum is one of rich nutrients liquid, so that a lot of bacteria grow on it especially LAB that



DOUBTFUL PROFILE	
Strip	API 50 CHB V4.0
Profile	-+-++++++--++++--------++++++++++--++------------
Note	

Significant taxa	% ID	T	Tests against			
Bacillus coagulans	93.7	0.46	DARA 4%	LXYL 0%		

Next taxon	% ID	T	Tests against			
Bacillus circulans	2.6	0.24	DARA 18%	LXYL 1%	MAN 89%	GLYG 92%
			GEN 98%	TUR 85%		

GOOD IDENTIFICATION	
Strip	API 50 CHB V4.0
Profile	--------+ --------++ ---------- ----------
Note	

Significant text	% ID	T	Test against			
Bacillus coagulans	99.1	0.48	MAE 9%	AFB 9%	ESC 9%	SAL 9%

Next taxon	% ID	T	Test against			
Bacillus circulans	0.40	0.22	GAL 0%	NAG 98%	MAL 88%	TRE 98%

Fig. 1: API test result of selected isolate

isolated and selected in this research. Two selected bacteria was potential as probiotic candidate, because the survival ability at low pH and high bile salt concentrations that similar with the gastrointestinal tract. The isolate expected to be through high gastric acidity and excretion of bile salt that could harmfull to the isolate (Salminen *et al*., 1999; Surono, 2004; Susanti *et al*., 2007).

CONCLUSION

Isolation and identification CFH colostrum bacteria that grown in NA enriched by olive oil resulting in 3 genus i.e., *Bacillus*, *Micrococcus* and *Staphylococcus*, meanwhile the isolate grown in NA enriched by 1% casein shown 6 genus i.e., *Bacillus*, *Staphylococcus*, *Micrococcus* and *Streptococcus* as gram positives strain, also *Enterococcus* and *Veillonella* as gram negative strain. Dominant genus that isolated was bacillus, so that two bacteria isolate was taken and identified with API test that resulting 93.7% similarity with *Bacillus coagulans* for Ic8 and 99.1% similarity with *Aneurinibacillus aneurinilyticus* for Ic12. Selected bacteria was potential as probiotic candidates because the survival ability at low pH and high bile sat concentrations.

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